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Retinol Inhibits the Growth and Invasion of *All-Trans*-Retinoic Acid Resistant Colon Cancer *in vitro* and *in vivo*

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Colorectal cancer is the third most common cancer and cause of death due to cancer in the United States. Death due to colorectal cancer is generally caused by hepatic metastasis rather than the primary tumor itself. The five-year survival rate is only 10% for patients whose colorectal cancer metastasized, which indicates the need for more effective therapies to treat colon cancer. The diet contains (1) preformed vitamin A as retinyl esters in animal-derived food sources and (2) provitamin A carotenoids in plant-derived food sources. Once absorbed, retinol is re-esterified and transported to the liver, the major site of vitamin A storage. Therefore, dietary vitamin A supplementation can increase retinol levels in the colon and liver, potentially affecting both primary colon tumors and liver metastases of the primary tumors.

All-trans–retinoic acid (ATRA) is thought to regulate most of the effects of retinoids, via the ATRA/RAR/RARE pathway exerting an inhibitory effect on cancer growth and progression. As cancer progresses, colon cancer acquires the resistance to

ATRA. The purpose of this study is to understand the mechanism by which retinol decreased the growth and progression of ATRA-resistant human colon cancer *in vivo* and *in vitro*. We first demonstrated that retinol decreased the growth of ATRA-resistant colon cancer cells by arresting cell cycle progression independent of the ATRA/RAR/RARE pathway. Next, we showed retinol inhibited ATRA-resistant human colon cancer cell invasion by decreasing MMP-2, -9 and PI3K activity *in vitro.* Finally, dietary vitamin A supplementation decreased the incidence and multiplicity of liver metastases in nude mice intrasplenically injected with ATRA-resistant human colon cancer cells. Taken together, these data suggest the possibility of dietary vitamin A supplementation for colon cancer therapy and prevention.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

1. COLORECTAL CANCER

1.1. Colorectal Cancer General Overview

Colorectal cancer is the third most common cancer and cause of death due to cancer in the United States. In 2007, an estimated 153,760 new cases are expected to be diagnosed, and 52,180 deaths from colorectal cancer are predicted to occur in 2007 (1). Genetic alterations are associated with colon carcinogenesis (Figure 1.1). Generally, five to seven deleterious genetic alterations occur when a normal epithelial cell becomes a carcinoma. There are two key pathways by which these genetic alterations can result in colon cancer: (1) chromosomal instability (CIN) and (2) microsatellite instability (MSI). About 85% of colorectal cancers are due to CIN and the remaining 15% are due to MSI (2). Colon cancer caused by CIN contains a variety of changes in chromosome number. It includes deleterious losses at chromosome 5q, chromosome 18q, and chromosome 17p. *KRAS*, *Adenomatous Polyposis Coli (APC) (5q)*, *DCC/MADH2/MADH4 (18q)*, and *TP53 (17p)* are important genes on these chromosomes. For example, the loss of *APC* is the first step in the colon cancer progression pathway. The *APC* gene, located on chromosome 5q, plays an important role in cell adhesion and signal transduction by regulating β-catenin degradation (3). Moreover, it is a tumor suppressor gene (4). The mutations of the APC gene include insertions, deletions, and nonsense mutations which can cause frame shifts and premature stop codons and are associated with Familial Adenomaceous Polyposis Coli (FAP) (5).

Colorectal cancer caused by MSI includes a series of defects in the DNA mismatch-repair genes. The mutations of the tumor suppressor genes as well as DNA mismatch-repair genes also lead to the transition from a normal cell into a tumor cell. Specifically, these mutations are associated with hereditary nonpolyposis colorectal cancer (HNPCC). These DNA repair genes include *hMSH2* (*human mutS homolog 2*) on chromosome 2p16, *hMLH1* (*human mutL homolog 1*) on chromosome 3p21, *PMS2* (*postmeiotic segregation 2*) on chromosome 7p22, and *hMSH6* on chromosome 2p16.

Risk of colon cancer increases with age. More than 90% of cases are diagnosed in people older than 50. The risk is also increased by certain acquired or inherited mutations as mentioned above, a personal or family history of colon cancer and polyps, or a personal history of inflammatory bowel disease. Several modifiable factors are associated with increased risk of colon cancer. These include obesity, physical inactivity, smoking, heavy alcohol consumption, a diet high in red or processed meat, and insufficient intake of fruits and vegetables (6).

1.2. Colon Cancer Metastasis

Five year survival rate for patients with colorectal cancer is 64% (1). If colorectal cancer is detected in early stage, the five year survival rate is 90% (1). However, only 39% of colorectal cancer is diagnosed at early stage because of the low rate of screening (1). Death due to colorectal cancer is generally caused by hepatic metastasis of the primary tumor, rather than the primary tumor itself (7). The five-year survival rate for colorectal cancer patients with metastasis is only 10% (1). Metastasis

consists of several processes including: (1) invasion or digestion of the basement membrane by tumor cells and migration of these cells through the basement membrane into the circulation (intravasation) and (2) migration of the cells out of the circulation and invasion into the target tissue (extravasation). Colorectal cancer is rated using Duke's classification system that contains four stages: A to D. Duke's stage A refers to tumors that affect only the mucosa of the bowel and not other structures of the colon. Duke's stage B indicates the tumor has invaded into or through the muscularis propria of the colon. In Duke's stage C the cancer has spread to the local lymph nodes. Duke's stage D indicates that the cancer has metastasized to distant tissues and organs.

Metastasis and Matrixmetalloproteinases (MMPs)

Cancer cell invasion and metastasis are multistep processes that involve extracellular matrix (ECM) proteolysis and changes in adhesion molecules. Matrix metalloproteinases (MMPs) are family of proteolytic enzymes which degrade the ECM during cancer progression [For a review please see: (8,9)]. MMP overexpression has been linked with invasion and metastasis and inhibitiors of MMPs block invasive and metastatic activities of many tumor types. MMP's are classified into four groups based on their substrate; collagenases, gelatinases, stromelysins and matrilysins. Particularly one group of MMPs, the gelatinases MMP-2 and –9, are associated with progression of colon cancer [For a review please see: (9)]. MMP-2 and -9 mRNA were elevated in colon cancer tissues as compared with normal colon tissues (10,11). Protein levels and activation of MMP-2 and -9 in colon cancer were closely related with Duke's staging

(12). The protein levels of MMP-2 and -9 were increased in invasive regions of colon cancer (13). Specifically, MMP-2 was significantly overexpressed in Duke's D stage (13,14). MMP-2 and -9 activation has also been shown to be increased in patients with metastases (15). Those data indicate that MMP-2 and -9 are involved in cancer progression.

Metastasis and Phosphatidylinositol 3-kinases (PI3K) Signaling

Phosphatidylinositol 3-kinases (PI3Ks) catalyze the phosphorylation of the 3-OH position of the inositol head groups of the phosphatidylinositol (PI) lipids, phosphatidylinos--itol(4)phosphate [PI(4)P], and phosphatidylinositol(4,5)bisphosphate [PI(4,5)P2] to generate PI(3)P, PI(3,4)P2 and PI(3,4,5)P3, respectively (16). PI(3,4)P2 and PI(3,4,5)P3 are generally absent from resting cells, but their intracellular concentration rises markedly upon stimulation via a variety of cell surface receptors, suggesting a second messenger function. PI3K can be classified into three main groups on the basis of their substrate and structure. The class-1 PI3K is the most studied and is a key enzyme in a intracellular signaling pathway, modulated by many growth and survival factors, which regulate cell proliferation, growth, survival and apoptosis (17,18). In addition, the class-1 PI3K is highly expressed in colon cancer cell lines (19). The class-1 PI3K is a heterodimer composed of a catalytic subunit (p110) and a regulator subunit (p85). The substrate for class I PI3K is PI(4,5)P2 which generates the second messenger PI(3,4,5)P3. The PI3K are also involved in cancer cell proliferation, survival, motility, differentiation, cytoskeletal rearrangement, and angiogenesis (20,21).

The PI3K gene is often overexpressed or mutated contributing to tumor progression in breast, colon, and several other cancers [For a review please see: (22)]. In colon cancer, *PIK3CA*, encoding the p110 subunit, is often mutated in either the kinase domain or helical domain to increase PI3K activity and consequently increase metastasis of colon cancer (23,24).

1.3. Dietary Chemoprevention of Colorectal Cancer

The five-year survival rate for colon cancer has dropped for the past 20 years because of improved diagnosis and treatment. However, the five-year survival rate is still 10% for patients whose colorectal cancer metastasized, which indicates the need for more effective therapies to treat colon cancer. Many studies have examined nutrients such as calcium, folate, fiber, omega-3 fatty acids, vitamin D, and vitamin A as prospective chemoprevention and chemotherapy agents for colorectal cancers [For a review please see; (25)]. Fiber has long been considered as a chemoprevention agent for colon cancer. However, intervention randomized trials failed to support the inverse effect of fiber intake on colon cancer prevention (26). However, the other nutrients mentioned above are promising in colon cancer prevention. For example there is an inverse association between calcium and vitamin D intake and risk of colon cancer (27). Calcium and vitamin D may act together to decrease the risk of colorectal cancer, as vitamin D is required for the absorption of calcium. Interestingly, folate showed two opposing effects depending on the stage of colon cancer. Folate supplementation had an inhibitory effect on the initiation of colon cancer progression (28). However, folate

deficiency reduced the size of colon cancer lesion (28). Omega-3 fatty acids in fish oil protect against colon tumorigenesis in epidemiologic, preclinical and clinical studies [For a review please see; (29)]. In addition, the retinoids, a group of compounds consisting of vitamin A (retinol), its natural metabolites, and several synthetic compounds have been shown in numerous experimental situations to act as cancer chemopreventive and therapeutic agents [for review see: (30-32)]. *All-trans*-retinoic acid (ATRA) accomplishes all these activities and most studies concerning retinoids have investigated the mechanisms by which ATRA regulates tumor growth and progression. However, tumors became ATRA-resistant as cancer progresses and the colon is exposed to retinol from the diet. Therefore, we evaluated the antiproliferative, antimetastatic, potentially chemotherapeutic, and chemopreventive effects of dietary vitamin A (retinol) on human colon cancer.

2. RETINOL

Retinol (Vitamin A), a fat-soluble vitamin, has important roles in vision, growth and development, immune function, and reproduction. The chemical structure of retinol contains a β-ionone ring, a polyunsaturated tail, and an alcohol end group (Figure 1.2). The diet contains (1) preformed vitamin A as retinyl esters in animal-derived food sources and (2) provitamin A carotenoids in plant-derived food sources. Retinyl esters are cleaved within the intestinal lumen to yield retinol (Figure 1.2). When retinol is absorbed from the enterocytes, retinol is re-esterified and transported to the liver via

chylomicrons (33). Retinol is de-esterified and excreted from the liver and circulates in the blood as a retinol binding protein (RBP)-retinol-transthyretin complex.

Retinol is dissociated from RBP and delivered to cells. The mechanism of retinol transport from plasma to cytoplasm is still unclear. Intracellularly, retinol is bound to cellular retinol binding protein (CRBP). There are three CRBPs; CRBP I, expressed in the liver, kidney and eye, CRBP II, mainly expressed in the small intestine, and CRBP III, expressed in the heart, muscle, adipose and mammary tissues [For a review please see:(34)]. Retinol is converted to either retinyl esters for storage by lethicin:retinol acyltransferase (LRAT) in the intestine or acyl-CoA-retinol acyltranserase (ARAT) in the liver, mammary gland, or adipose tissue or to retinaldehyde as an intermediate form before metabolism to ATRA by alcohol dehyrogenase (ADH) or retinol dehydrogenase (ROLDH). Retinaldehyde is further metabolized to ATRA by retinal dehydrogenase (RALDH) (Fig. 1.2). CRABP I catalyzes the degradation of ATRA to *all-trans*-4-oxo-retinoic acid to lower the active intracellular ATRA concentrations (35). On the other hand, CRABP II transports ATRA to the nucleus where it interacts with retinoic acid receptors (RAR).

The action of ATRA is mediated by nuclear RAR and retinoid X receptor (RXR), each consisting of three receptor types, α, β and γ. The heterodimer of RAR and RXR with ligand regulates ATRA-mediated gene transcription by binding to retinoic acid response elements (RAREs) [for review see: (36)]. RAREs are *cis*-acting elements in the promoter regions of retinoid-responsive genes composed of direct repeats (DR) of the consensus half-site sequence AGGTCA separated by five nucleotides. For example,

ATRA induces the expression of the RARβ through this mechanism regulating cell proliferation, growth and survival (37,38).

3. COLON CANCER AND RETINOL

As mentioned above, ATRA is thought to regulate most of the effects of retinoids, via the ATRA/RAR/RXR/RARE pathway. Unfortunately, the period of inhibitory effect of ATRA on colon cancer is short due to acquisition of resistance to ATRA. ATRA resistance frequently occurs during cancer progression. ATRA-resistance is due to a defect in RAR α, β, or γ induction in response to ATRA [for review see: (31,39-41)]. The defective receptor varies with cell line but RARβ expression is frequently lost because of methylation of the RARE on the RARβ promoter region (39-42).

Dietary retinyl-esters are converted to retinol in the intestinal lumen. The intestinal lumen, including colonocytes, is primarily exposed to retinol. Thus, dietary vitamin A supplementation can elevate retinol levels in the colon. Once absorbed, retinol is esterified and transported to the liver, the major site of vitamin A storage. Although serum retinol levels in non-vitamin A deficient animals vary from 1-2 μM, regardless of supplementation status, [for a review please see: (43)], hepatic retinol levels increase in response to supplementation and values in excess of 90 μM have been reported (44). Therefore, retinol could potentially be used to treat not only primary colon tumor growth but also liver metastases.

Retinoids have been shown in numerous experimental situations to act as cancer chemopreventive and therapy agents [for review see: (30-32)]. Retinol, 9-*cis*-retinoic

acid (*9-cis*-RA), and 4-(hydroxyphenyl)retinamide (4-HPR) can inhibit the formation of carcinogen-induced aberrant crypt foci, a precursor to colon cancer, as well as colon tumors in rats (45-48). Retinyl palmitate was recently shown to inhibit high fat diet-induced aberrant crypt foci (45). In addition, several *in vitro* studies illustrate that retinoids have potent antiproliferative effects on colon cancer cell lines and may hold potential for both chemoprevention and chemotherapy of colon cancer. Taken together, it is more relevant to examine the effects of retinol on colon cancer cell growth and metastasis because (1) colonocytes are primarily exposed to retinol, (2) liver is the major storage site of retinol and colon cancer metastasis site, and (3) ATRA resistance is common in colon carcinomas.

This dissertation focuses on the study of retinol as an inhibitor of ATRA-resistant human colon cancer cell line growth and metastasis *in vitro* and *in vivo*. Chapter 1 introduces background information concerning colorectal cancer progression and the role of retinol as a chemotherapy agent for colon cancer. Chapter 2 will focus on the initial studies showing the mechanism of growth inhibition in ATRA-resistance colon cancer cells by retinol treatment independent of ATRA/RAR/RARE signaling. Chapters 3 and 4 are concerned with the ability of retinol to inhibit colon cancer cell invasion by decreasing MMP-2 and -9 activity as well as PI3K activity, respectively. Chapter 5 will focus on the ability of dietary vitamin A supplementation to inhibit the liver metastasis of colon tumors in a nude mouse xenograft model. Chapter 6 will summarize the findings of these studies and propose future directions to continue to understand the molecular mechanisms by which retinol inhibits the growth and metastasis of ATRA-resistant human colon cancer cell lines.

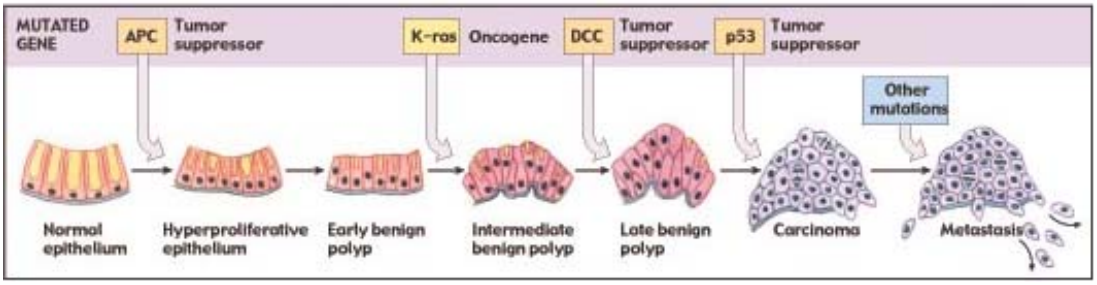

FIGURE 1.1. GENETIC ALTERATIONS ASSOCIATED WITH COLON CARCINOGENESIS. Mutation in APC, K-ras, DCC and p53 transform normal colon epithelium to metastatic colon cancer (49).

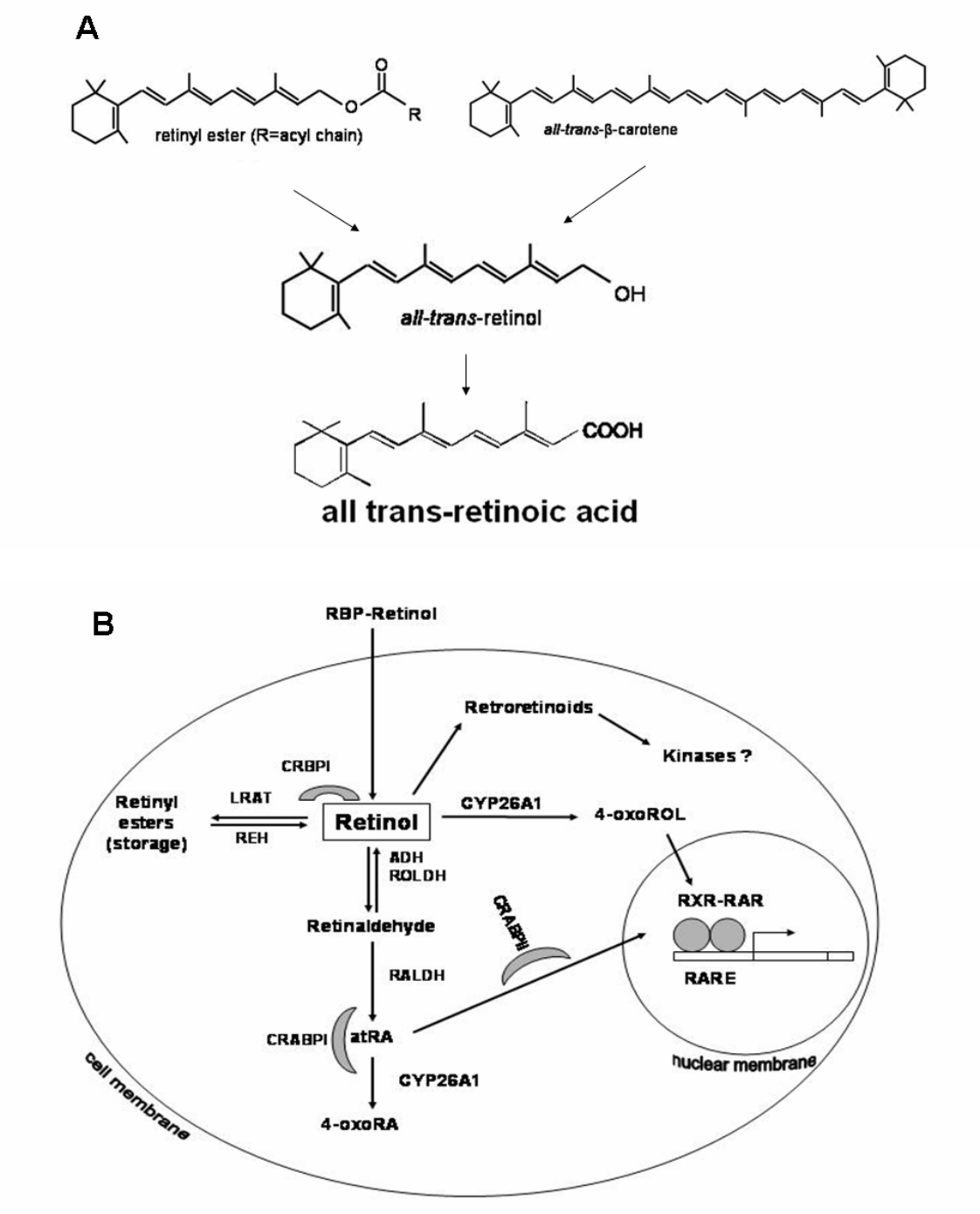

FIGURE 1.2. A. RETINOID METABOLISM (34) AND B. SIGNALING VIA RAR, RXR AND RARE

Chapter 2: Retinol Inhibits the Growth of All-Trans-Retinoic Acid-Sensitive and -Resistant Colon Cancer Cells through a Retinoic Acid Receptor-Independent Mechanism

ABSTRACT

Retinol (vitamin A) is thought to exert its effects through the actions of its metabolite, *all-trans*-retinoic acid (ATRA), on gene transcription mediated by retinoic acid receptors (RAR) and retinoic acid response elements (RARE). However, RA-resistance limits the chemotherapeutic potential of ATRA. We examined the ability of retinol to inhibit the growth of ATRA-sensitive (HCT-15) and ATRA-resistant (HCT-116, SW620, and WiDr) human colon cancer cell lines. Retinol inhibited cell growth in a dose-responsive manner. Retinol was not metabolized to ATRA or any bioactive retinoid in two of the cell lines examined. HCT-116 and WiDr cells did convert a small amount of retinol to ATRA, however this amount of ATRA was unable to inhibit cell growth. To show that retinol was not inducing RARE-mediated transcription, each cell line was transfected with pRARE-CAT (chloramphenicol acetyltransferase) and treated with ATRA and retinol. Although treatment with ATRA did increase CAT activity five-fold in ATRA-sensitive cells, retinol treatment did not increase CAT activity in any cell line examined. To demonstrate that growth inhibition due to retinol treatment was independent of ATRA, RAR, and RARE, a pan RAR-antagonist was used to block RAR-signaling. Retinol-induced growth inhibition was not alleviated by the RAR-antagonist

in any cell line, but the antagonist did alleviate ATRA-induced growth inhibition of HCT-15 cells. Retinol did not induce apoptosis, differentiation or necrosis, but did affect cell cycle progression. Our data show that retinol acts through a novel, RAR-independent mechanism to inhibit colon cancer cell growth.

INTRODUCTION

Colorectal cancer is currently the third leading cause of death due to cancer in the United States. Retinoids, a group of compounds consisting of vitamin A (retinol), its natural metabolites, and several synthetic compounds, have been shown to act as cancer chemopreventive agents [for reviews see: (30-32)]. Retinol, *9-cis*-retinoic acid (*9-cis*-RA), and 4-(hydroxyphenyl)retinamide (4-HPR) can inhibit the formation of carcinogen-induced aberrant crypt foci, a precursor to colon cancer, as well as colon tumors in rats (45-48). Retinyl palmitate was recently shown to inhibit high fat diet-induced aberrant crypt foci (45). In addition, several *in vitro* studies illustrate that retinoids have potent antiproliferative effects on colon cancer cell lines and may hold potential for both chemoprevention and chemotherapy of colon cancer.

In almost all of the above studies, the retinoid examined has been an isoform of RA or a synthetic retinoid such as 4-HPR. Although these compounds are effective at inhibiting *all-trans*-RA (ATRA)-sensitive cell growth, the use of exogenous ATRA to study the effects vitamin A assumes that all of the biological phenomena attributed to retinol are due to ATRA. The diet contains very little ATRA (33). Rather, the diet contains vitamin A as: (1) previtamin A carotenoids and (2) preformed vitamin A as

retinol and retinyl esters. Retinyl esters are cleaved within the intestinal lumen to yield retinol. Therefore, human colonocytes are exposed primarily to retinol, the focus of this study. Within most cells, retinol is either esterified for storage or metabolized to ATRA. ATRA effects cell growth and differentiation by binding to retinoic acid receptors (RARs), located in cell nuclei. RARs heterodimerize with retinoid X receptors (RXRs) and bind to retinoic acid response elements (RAREs) located in the regulatory regions of retinoid-responsive genes. When ATRA binds to the RAR member of the RAR/RXR heterodimer, gene transcription via RARE is induced [for review see: (31)].

RA-resistance is believed to be due to a defect in RAR α, β, or γ induction in response to ATRA [for review see: (31,39-41)]. RA-resistance occurs when tumors or tumor-derived cell lines cease to growth inhibit or differentiate in response to treatment with ATRA. Retinoic acid resistance is a common phenomenon and appears to arise spontaneously in numerous types of cancer and tumor-derived cell lines. The defective receptor varies with cell line but RARβ expression is frequently lost.

The objective of the present study was to determine if retinol inhibits the growth of both ATRA-sensitive and ATRA-resistant colon cancer cell lines *in vitro*. Because the ATRA-resistant cell lines lack one or more RARs, their use allowed us to determine the effects of retinol on cell growth, exclusive of the effects of ATRA. Our data show that retinol itself inhibits the growth of both ATRA-sensitive and ATRA-resistant colon cancer cells through an ATRA and RAR independent mechanism.

MATERIALS AND METHODS

Tissue Culture

Three human colorectal adenoma cell lines, HCT-15, SW620, and WiDr, and one human colon carinoma cell line, HCT-116, were obtained from the American Type Culture Collection (Manassas, VA) and grown as recommended. HCT-15 cells were grown in MEM, HCT-116 in McCoy's medium, and SW620 and WiDr cells in DMEM in a humidified atmosphere at 37°C with 5% CO_2. All medium was supplemented with 10% FCS (fetal calf serum) and antibiotics (1000 U/mL penicillin and 1000 μg/mL streptomycin). The experiment was repeated with each cell line grown in either supplemented DMEM or McCoy's medium. Medium type did not affect cell growth. Cells were seeded in 12-well culture dishes at a density of 1 x 10^4 cells per well. The following day the medium was removed and replaced with medium containing 0, 0.1, 1 or 10 μM ATRA or *all-trans*-retinol (Sigma, St Louis, MO). All retinoids were prepared as 10 mM stocks in 100 % ethanol. All treatments, including control, received equal volumes of ethanol vehicle and all retinoid manipulations were performed under subdued lighting. All treatments were performed in duplicate. Cells were harvested using trypsin and counted via hemocytometer every 24 h for four days.

Retinoid Extraction and HPLC Analysis.

To examine retinol metabolism, cells were seeded in 60 mm dishes at the following densities to yield 60 to 80% confluency and maximum HPLC detection

sensitivity at the time of harvest: 5 x 10^5 cells/dish for 24 h, 2.5 x 10^5 cells/dish for 48 h, 1 x 10^5 cells/dish for 72 h, and 5 x 10^4 cells/dish for 96 h. Twenty-four hours after plating, cells were treated with 0, 1, and 10 μM retinol for 24, 48, 72 or 96 h. Sixteen hours before harvest, the culture medium was removed and replaced with medium containing 5% FCS and 50 nmol/L [^{3}H]retinol (specific activity = 52.5 Ci/mmol). Cells and medium were harvested 2, 4, 8, and 16 h after the addition of label as described previously (50). A control of labeling medium without cells was also incubated for 16 h. F9 murine teratocarcinoma cells, treated with 1 μM ATRA for 48 hr and incubated with 50 nM [^{3}H]-retinol for 16 hr were used as a positive control for 4-oxoretinol production (17). Retinoids were extracted and separated using a Waters Millennium HPLC system as described previously (51).

Cell Transfection and CAT Assays.

To examine the possibility that an undetected metabolite of retinol was activating RAR/RXR-mediated transcription, all cell lines were transiently transfected with pRARE-CAT (generously provided by Dr. Dianne Soprano, Temple University, Philadelphia, PA). Cells were seeded on to 12-well plates at a density of 1.75 X 10^5 cells/well, and incubated overnight in FCS-supplemented medium. The following day cells were transfected using Lipofectamine 2000 (Promega, Madison, WI) according to the manufacturer's protocol with 1 μg of pRARE-CAT and 0.5 μg of pSV-β-gal. Twenty-four hours later the transfection medium was removed and the cells were treated with fresh medium containing 0, 1, and 10 μM ATRA or retinol. The cells were

harvested after treatment for 24 or 48 h and assayed for β-galactosidase (β-Galactosidase Enzyme Assay System, Promega, Madison, WI) and CAT (CAT Enzyme Assay System, Promega, Madison, WI) activity as per manufacturer's instructions. CAT activity was corrected for transfection efficiency using the β-galactosidase activity.

RAR Antagonist Assays.

To determine if retinol was inhibiting cell growth via RAR, the pan-RAR antagonist, AGN 193109 was used to block RAR function. The RAR pan-antagonist was synthesized by Allergan, Inc. (Irvine, CA). Cell lines were plated at a density of 1 x 10^4 in 12-well plates and allowed to attach overnight. The following day, HCT-15 cells were treated with 0 and 1 μM ATRA or retinol with and without 10 μM AGN 193109. HCT-116, SW620, and WiDr cells were treated with 0 and 1 μM retinol with and without 10 μM AGN 193109. Control cells received an equal volume of DMSO and ethanol vehicle. Cells were harvested after treatment for 48 h (HCT-15) or 96 h (HCT-116, SW620, and WiDr). All treatments were performed in duplicate. The pharmacological, 10 μM, concentrations of ATRA and retinol were not examined because 100 μM AGN 193109 was toxic to the cells.

Detection of Apoptosis.

Nuclear staining via DAPI (4, 6-diamidino-2-phenylindole) and flow cytometry analysis, described below, were used to determine if cell growth inhibition was due to apoptosis. For DAPI staining, cells were plated at 1 x 10^4 cells per well in 12-well

plates before treatment with 0, 1, and 10 μM retinol. Cells incubated for 4 hr at 37ºC with 4 μg/mL camptothecin served as the positive control for apoptosis. Both adherent and floating cells were harvested every 24 h for 4 d. The cells were centrifuged and washed with PBS to remove all traces of media. Cells were then incubated with 2 μg/mL DAPI for 10 min at 37ºC before counting at 400X magnification with an Olympus upright fluorescence microscope. To obtain cell counts, at least three different locations on each slide were used. Two hundred cells were counted at each location yielding a minimum of 600 cells counted per slide. Cells with segmented nuclei were scored as apoptotic.

Cellular Differentiation.

Alkaline phosphatase activity was used to determine if retinol was inhibiting cell growth by inducing cellular differentiation. All cell lines were plated on 60 mm dishes at a density of 5 x 10^4 cells per plate. Twenty-four hours later, cells were treated with 0, 1 and 10 μM retinol or 2 mM sodium butyrate (positive control) for 96 h. Alkaline phosphatase activity was determined as described previously (52). Alkaline phosphatase activity was measured by the conversion of p-nitrophenyl phosphate (19.8 mM) to p-nitrophenol by 0.1 mL of cell lysate in 100 mM glycine buffer, containing 1 mM $MgCl_2$, pH 10. Alkaline phosphatase enzyme activity was corrected for lysate protein content and expressed as percent positive control.

Necrosis assays.

Trypan blue exclusion assays were performed to measure cell death. Briefly, an aliquot of the cells harvested for the growth curve assays was pelleted by centrifugation, resuspended in 0.5 mL HBSS, and incubated with an equal volume of 0.4% trypan blue solution (Sigma-Aldrich, St. Louis, MO) for five minutes at room temperature before counting with a hemocytometer. Blue cells were scored as necrotic.

Flow Cytometry Analysis.

To determine if growth inhibition was due to cell cycle arrest and to confirm the absence of apoptosis through lack of a sub-G_1 peak, cells were seeded on 60 mm dishes at a density of 3 x 10^5 (HCT15 and SW620) or 2 x 10^5 (HCT-116 and WiDr) cells per dish to provide 50-60% confluence at the time of harvest. To synchronize, cells were plated in serum-free medium for 24 h and treated with 1 (HCT-15) or 3 μg/mL (HCT-116, SW620, WiDr) aphidicholin for an additional 24 hr. The following day, the cells were washed with PBS and treated with fresh FCS-supplemented media containing 0, 1, and 10 μM retinol. Cells were harvested, fixed in 70% ethanol overnight and stained with 40 μg/mL propidium iodide as described previously (Ormerod). At least 10,000 cells were analyzed per sample using a FACSCalibur machine (Becton Dickinson, San Jose, CA). DNA content was determined using Modfit software version 3.0 (Verity Software House, Inc, Topsham, ME).

RESULTS

Growth of ATRA-resistant colon cancer cells is inhibited by retinol.

The ability of retinol to inhibit cell growth was examined in three ATRA-resistant human colon cancer cell lines HCT-116 (42), SW620 (53), and WiDr (39). HCT-15, an ATRA-sensitive cell line, was chosen to serve as a positive control for the inhibitory effects of ATRA on colon cancer cell growth (54). Serum concentrations of retinol range from 0.5 to 2 μM (55). Therefore, 0.1 μM was selected to represent a sub-physiological, and 1 μM a physiological, concentration of retinol. The highest level, 10 μM retinol, was used as a pharmacological, but potentially therapeutically relevant, concentration. There is very little ATRA (4-14 nM) in the serum (56,57). ATRA levels were chosen to match the concentrations of retinol used and to reflect ATRA levels commonly found in the literature (39-41,58).

After 96 h of treatment, the growth of HCT-15 cells was inhibited by ATRA (Figure 2.1A), as expected. In addition, HCT-15 cell growth was also inhibited by retinol in a dose-responsive manner. Cells treated with 10 μM retinol exhibited the largest degree of inhibition to 36.7 ± 7.8 % of control. HCT-116 cell growth was inhibited slightly by 0.1 and 1 μM retinol and this decrease was not significant when compared to the same concentrations of ATRA (Figure 2.1B). However, HCT-116 cell growth was significantly inhibited by 10 μM retinol (37.5 ± 9.2 % of control) when compared to 10 μM ATRA (74.3 ± 4.7 % of control). SW620 and WiDr cell growth was significantly inhibited by treatment with 0.1 and 1 μM retinol for 96 h when compared to ATRA (Figure 2.1C and D) indicating that physiological levels of retinol

can inhibit the growth of ATRA-resistant cells. At 10 μM concentrations, there was no significant difference in the ability of ATRA and retinol to inhibit SW620 and WiDr cell growth. The highest concentration of ATRA, 10 μM, inhibited cell growth slightly in all cell lines examined (Figure 2.1). These data show that retinol can inhibit the growth of both ATRA-sensitive and ATRA-resistant colon cancer cells.

Retinol is not metabolized to bioactive compounds.

To determine if retinol was metabolized to a bioactive compound, such as ATRA, anhydroretinol (AR), or 4-oxoretinol, reverse-phase HPLC was performed on all cell lines as described previously (51). To ensure that no transient bioactive retinoids were overlooked, retinol metabolism was examined after 24, 48, 72 and 96 h of treatment with 0, 1, and 10 μM retinol followed by 2, 4, 8, and 16 h of incubation with 50 nM [^{3}H]-retinol.

HCT-15 and SW620 cells produced no [^{3}H] -ATRA, 4-oxoretinol, or AR from [^{3}H]-retinol at any time point or treatment (Figure 2.2). The absence of [^{3}H]-4oxoretinol was also confirmed by northern blot analysis that failed to show CYP261A mRNA expression in any of the four cell lines examined (data not shown). HCT-116 and WiDr cells did synthesize a small amount of [^{3}H]-ATRA from [^{3}H]-retinol (Figure 2.2). However, both control and retinol-treated cells metabolized [^{3}H]-retinol to [^{3}H]-ATRA. The time point displayed in Figure 2.2 (48 h of retinol treatment followed 8 h of incubation with 50 nM [^{3}H]-retinol) showed the largest concentration of 50 nM [^{3}H]-ATRA synthesis by HCT-116 and WiDr cells of any time point examined. When

corrected for cell number, HCT-116 cells treated with the vehicle control synthesized 0.15 nM [^{3}H]-ATRA/million cells from 50 nM [^{3}H]-retinol. Cells treated with 10 μM retinol synthesized 0.55 nM [^{3}H]-ATRA/million cells from 50 nM [^{3}H]-retinol. Because the metabolism of 50 nM [^{3}H]-retinol reflects the metabolism of 10 μM retinol (50,51,59), we can assume that if HCT-116 cells were treated with 10 μM retinol, 0.11 μM of ATRA would be produced per million cells. There were 5.4 x 10^6 cells on a duplicate plate of HCT-116 cells treated with 10 μM retinol for 96 h and included in the experiment shown in Figure 2.2. As can be seen in Figure 2.1B, HCT-116 cell growth is inhibited only slightly by 0.1 or 1 μM ATRA when compared to control. The amount or [^{3}H]-ATRA synthesized from [^{3}H]-retinol by WiDr cells was even less then that synthesized by the HCT-116 cell line. Therefore, the small amount of ATRA produced by these cell lines when treated with 10 μM retinol cannot be responsible for the decrease in cell number that occurs when these cells are treated with 10 μM retinol.

Retinol does not induce RARE-CAT reporter gene expression.

Each colon cancer cell line was transiently transfected with pRARE-CAT and treated with ATRA or retinol to confirm that retinol was not metabolized to a bioactive compound that could transactivate RARE-mediated gene transcription. The pRARE-CAT construct contains only the nucleotides corresponding to the RARE found in the regulatory region of the RARβ2 gene linked to a CAT promoter.

ATRA-sensitive HCT-15 cells show that treatment of with both 1 and 10 μM ATRA for 48 h resulted in an increase in CAT activity to 4.98 ± 0.39 -fold over control at

48 h (Figure 2.3A). Because HCT-15 cells were ATRA-sensitive we were surprised to find that treatment of HCT-15 cells with retinol did not increase CAT activity to more then 1.80 ± 0.06 -fold over control at 48 h for cells treated with 1 μM retinol (Figure 2.3A). However, this lack of CAT activity reflects the metabolism data (Figure 2.2A-D) showing that HCT-15 cells do not metabolize retinol to ATRA.

Neither ATRA nor retinol increased CAT activity more then 1.7-fold over control in any of the three ATRA-resistant colon cancer cell lines (Figure 2.3B-D). The lack of CAT activity in cells treated with ATRA confirms the inability of these cells to respond to ATRA via RAR/RARE mediated mechanisms as described previously (39,42,53). Although the HCT-116 cells converted a small amount of [^{3}H]retinol to [^{3}H]ATRA, these cells lack RAR (42). The absence of an increase in CAT activity in response to retinol treatment in the HCT-116 and WiDr cell lines shows that the small amount of ATRA produced by these cells does not induce RAR/RARE-mediated gene transcription. SW620 cells did not metabolize [^{3}H]retinol to [^{3}H]ATRA (Figure 2) and the lack of CAT activity in SW620 cells when treated with retinol both confirms the metabolism data and shows that an RAR-activating metabolite of retinol is either not present or is incapable of activating RAR/RARE-mediated gene transcription. In summary, the inability of retinol to increase CAT activity in any of the cell lines examined, including ATRA-sensitive HCT-15 cells, demonstrates that retinol is not inducing RA-mediated gene transcription, confirming our metabolism data, and indicating that retinol may be acting exclusive of the RAR to inhibit colon cancer cell growth.

Retinol is not acting through the RAR to inhibit cell growth.

To confirm that the growth inhibition exhibited by cells treated with retinol was not mediated by the RA/RAR/RARE retinoid signaling mechanism, all cell lines were treated with a RAR pan-antagonist, AGN 193109. This antagonist, when added at 10-times the concentration of agonist, blocks the ability of agonist to bind to RAR (60). HCT-15 cells treated with 1 μM ATRA and 10 μM AGN 193109 served as a positive control for the ability of AGN 193109 to block RAR-mediated cell growth inhibition. Because 1 μM ATRA does not inhibit HCT-116, SW620, or WiDr cell growth we did not test the effects of the combined treatment of AGN 193109 and ATRA in these cell lines. As shown in Figure 2.4A, AGN 193109 blocked ATRA-induced growth inhibition in HCT-15 cells, as expected. However, AGN 193109 did not block growth inhibition due to retinol treatment in any of the four cell lines examined, including the ATRA-sensitive HCT-15 cell line (Figure 2.4). The inability of AGN 193109 to block retinol-induced growth inhibition confirms the results of the metabolism and RARE-reporter experiments which also indicate that retinol is not acting via RA/RAR/RARE to affect cell growth even in the ATRA-sensitive, HCT-15 cell line. Unlike the ATRA-resistant cell lines, HCT-15 cells contain all of the cellular machinery required for induction of ATRA/RAR/RARE-mediated gene transcription and growth inhibition (39). As shown in Figure 2.4A, ATRA is acting via this mechanism to inhibit the growth of HCT-15 cells. In contrast, retinol is acting via a novel, receptor-independent mechanism to inhibit the growth of both ATRA-resistant and surprisingly, ATRA-sensitive human colon cancer cell lines.

Retinol does not induce apoptosis, differentiation, or necrosis in ATRA-resistant colon cancer cells.

To determine the mode by which retinol inhibits the growth of colon cancer cells, apoptosis was examined by nuclear staining using DAPI (Figure 2.5, left column) and FACS analysis of DNA content (Figure 2.6). The percentage of DAPI-stained cells exhibiting segmented nuclei was less then 10% in all cell lines at all time points and treatments examined. FACS analysis failed to detect a sub-G_1 peak in any of the cell lines when treated with retinol, confirming the absence of apoptosis (Figure 2.6). Additionaly, in all cell lines, less than 4% apoptosis was detected with TUNEL assay and no apoptosis was detected by PAR-P cleavage or DNA laddering (data not shown). Therefore, retinol does not inhibit colon cancer cell growth by inducing apoptosis.

Alkaline phosphatase assays were performed to determine if retinol was inhibiting colon cancer cell growth by inducing cellular differentiation. Retinol does not induce alkaline phosphatase activity in HCT-116, SW620, or WiDr cells (Figure 2.5, right column). Retinol increased alkaline phosphatase activity slightly in HCT-15 cells (Figure 2.5A). In contrast, treatment with sodium butyrate resulted in a large increase in alkaline phosphatase activity in each cell line. These data indicate that retinol is not inhibiting cell growth by inducing cellular differentiation in the three ATRA-resistant cell lines. A small increase in alkaline phosphatase activity in HCT-15 cells treated with retinol may indicate that cellular differentiation accounts for part of the retinol-induced decrease in growth.

To ensure that retinol was not inducing necrosis, trypan blue dye exclusion assays were performed on the adherent cells used for the growth curve experiments described in

Figure 2.1. The percent of cells that stained with trypan blue dye varied between 0.1 and 7% and no consistent pattern was exhibited under any treatment condition at any time point (data not shown). Therefore, necrosis is not responsible for the growth inhibition exhibited by colon cancer cells treated with retinol.

Retinol affects cell cycle progression.

Treatment with 10 μM retinol increased the percentage of cells in $G_{0/1}$ while decreasing the percentage of cells in S-phase in the HCT-15, SW620, and WiDr cell lines (Figure 2.6 A, C, and D). Treatment with retinol decreased the percentage of HCT-15 cells in $G_{2/M}$, slightly increased the percentage of HCT-116 and SW620 cells in $G_{2/M}$, and notably increased the percentage of WiDr cells in $G_{2/M}$. In contrast, the percentage of HCT-116 cells in $G_{0/1}$ was not affected by retinol (Figure 2.6B), but retinol did decrease the percentage of HCT-116 cells in S-phase. As can be seen in Figure 2.1D, control HCT-116 cells continued to divide in a linear manner, while HCT-116 cells treated with 10 μM retinol ceased to divide between 24 and 48 h of treatment. This result, when considered in light of the absence of apoptosis, differentiation, and necrosis in the HCT-116 cell line despite strong growth inhibition by retinol, may indicate that retinol acts to slow the overall rate of cell division and increase the generation time of this cell line.

DISCUSSION

This study shows that retinol inhibits the growth of both ATRA-sensitive and ATRA-resistant human colon cancer cell lines. We provide three lines of evidence that

retinol is acting independent of the established ATRA/RAR/RARE retinoid signaling pathway. The first line of evidence indicates that retinol is not metabolized to bioactive compounds, such as ATRA, in two out of the four cell lines examined. The remaining two cell lines synthesized only small amounts of ATRA from retinol. Second, we show that retinol does not activate RARE-mediated gene transcription. Finally, we present evidence that a RAR-antagonist blocks the ability of ATRA to inhibit the growth of ATRA-sensitive HCT-15 cells, as expected, but does not block the ability of retinol to inhibit the growth of any cell line examined. The most surprising outcome of this study is that retinol is not acting through a RAR-dependent pathway in ATRA-sensitive HCT-15 cells. Therefore, even in the presence of functioning RAR, retinol does not inhibit cell growth by the actions of its metabolite ATRA, because this metabolite is not present in ATRA-sensitive HCT-15 cells (Figure 2.2).

The ability of retinol to inhibit colon cancer cell growth is particularly interesting given that colon cancer cell lines produce little or no ATRA (Figure 2.2). This finding is supported in a recent study by Jette *et al* (61) that used northern blot analysis to show that colon cancer cell lines, including HCT-116, lack retinol dehydrogenases, and therefore the ability to synthesize ATRA. The metabolism of retinol by colon cancer cells was not examined in the study by Jette *et al* (61). In contrast, our data shows that the HCT-116 cell line is capable of synthesizing very small amounts of ATRA from retinol (Figure 2.2). This discrepancy is perhaps due to the ability of HPLC to detect extremely small amounts of ^{3}[H]-retinoids, compared to the relative lack of sensitivity of northern blot analysis.

4-Oxoretinol and AR are two naturally occurring retinoids capable of inhibiting cell growth. 4-Oxoretinol acts via RARs (17), much like ATRA, whereas AR acts via a receptor-independent cytosolic mechanism to inhibit cell growth (62,63). Neither compound was formed from retinol by any of the cell lines we examined (Figure 2.2). We cannot eliminate the possibility that an unknown bioactive metabolite of retinol was formed that existed only briefly or was not detected by our HPLC protocol. However, the metabolism data is supported by the inability of retinol to induce CAT-activity in cells transfected with a pRARE-CAT construct (Figure 2.3) as well as the inability of a pan-RAR antagonist to block the effects of retinol on cell growth (Figure 2.4).

We chose to use the pan-RAR antagonist, AGN 193109 to block RARs because this compound exhibits a high affinity for RAR (60). Although a genetic approach would have been more specific, the dominant negative RAR construct available is activated by retinol (64), making it inappropriate for this study. Therefore, we included a positive control, showing that AGN 193109 blocks ATRA-induced growth inhibition in the HCT-15, ATRA-sensitive cell line (Figure 2.3*A*), to indicate that AGN 193109 is functioning to block RAR-mediated growth inhibition.

Retinoids have been previously shown to inhibit cancer cell growth by increasing cellular differentiation, inducing apoptosis, or causing cell cycle arrest. With respect to colon cancer, retinoids tend to induce tumor apoptosis both *in vitro* (39,65,66) and *in vivo*(47,67). The cell lines examined in this study showed no apoptosis in response to retinol treatment (Figure 2.5 left column, Figure 2.6). In contrast, retinol induced $G_{0/1}$ arrest in three of the cell lines examined (Figure 2.6). Although retinol failed to increase the percentage of cells in $G_{0/1}$ in the HCT-116 cell line, growth inhibition in

these cells could be due to an overall increase in generation time because retinol does decrease cell growth (Figure 2.1B). The differing responses between the HCT-116 cell line and the other three may reflect the heterogenicity of these cell lines, tumor stage (carcinoma versus adenoma), and presence or absence of various proteins in each cell line, for example APC (adenomatous polyposis coli) or p53.

Retinoids tend to induce cell cycle arrest by blocking the G_1 to S phase transition [for review see: (30)]. Unfortunately, the effect of retinoids on cell cycle regulatory proteins appears to be cell type specific (30). For example, in carcinogen-exposed immortalized human bronchial epithelial cells, ATRA-induced G_1 arrest is associated with decreased cyclin D1 protein levels due to ubiquitin-mediated degradation of cyclin D1 (68,69). In contrast, ATRA-induced G_1 arrest in MCF-7 breast cancer cells is associated with decreased pRB phosphorylation, while cyclin D1, $p21^{WAF1/CIP1}$, cdk4 and cdk6 activity either does not change or decreases slightly, depending on the study (70-72).

This study shows that retinol is acting independent of the RAR to inhibit colon cancer cell growth. Previously, retinoids have been shown to exert their receptor-independent effects via interactions with protein kinase C alpha (PKCα) (73), F-actin (74), c-Raf kinase (62), regulating mitochondrial membrane potential (75), generating reactive oxygen species (76), increasing intracellular ceramide levels (77), activating c-Jun N-terminal kinase (78), inducing ubiquitin-dependent proteolysis (68,69), and affecting MAP kinase (79,80), phosphatidylinositol 3-kinase (PI3K)/Akt (81), and epidermal growth factor receptor (EGFR) signaling (82). The Hammerling lab has shown that the retroretinoids, retinol, and ATRA can bind PKCα and affect its redox

activation (73). In contrast to our present study, they speculate that retinol antagonizes AR and increases cell survival by binding to c-Raf and augmenting its response to reactive oxygen species generated during UV irradiation, however the link between cell growth and c-Raf activation was not directly examined (62). Because AR induces apoptosis we do not expect retinol to be affecting cell growth by interacting with c-Raf or any of the other pathways listed above that induce apoptosis.

In conclusion, this study shows that retinol acts through a novel mechanism to inhibit the growth of both ATRA-sensitive and ATRA-resistant colon cancer cells by affecting cell cycle progression. Resistance to ATRA is a common phenomenon and limits the use of RA-derivatives as chemotherapy. We speculate that retinol, or a derivative of it, may prove an effective therapy to treat colorectal cancer.

ACKNOWLEDGEMENTS

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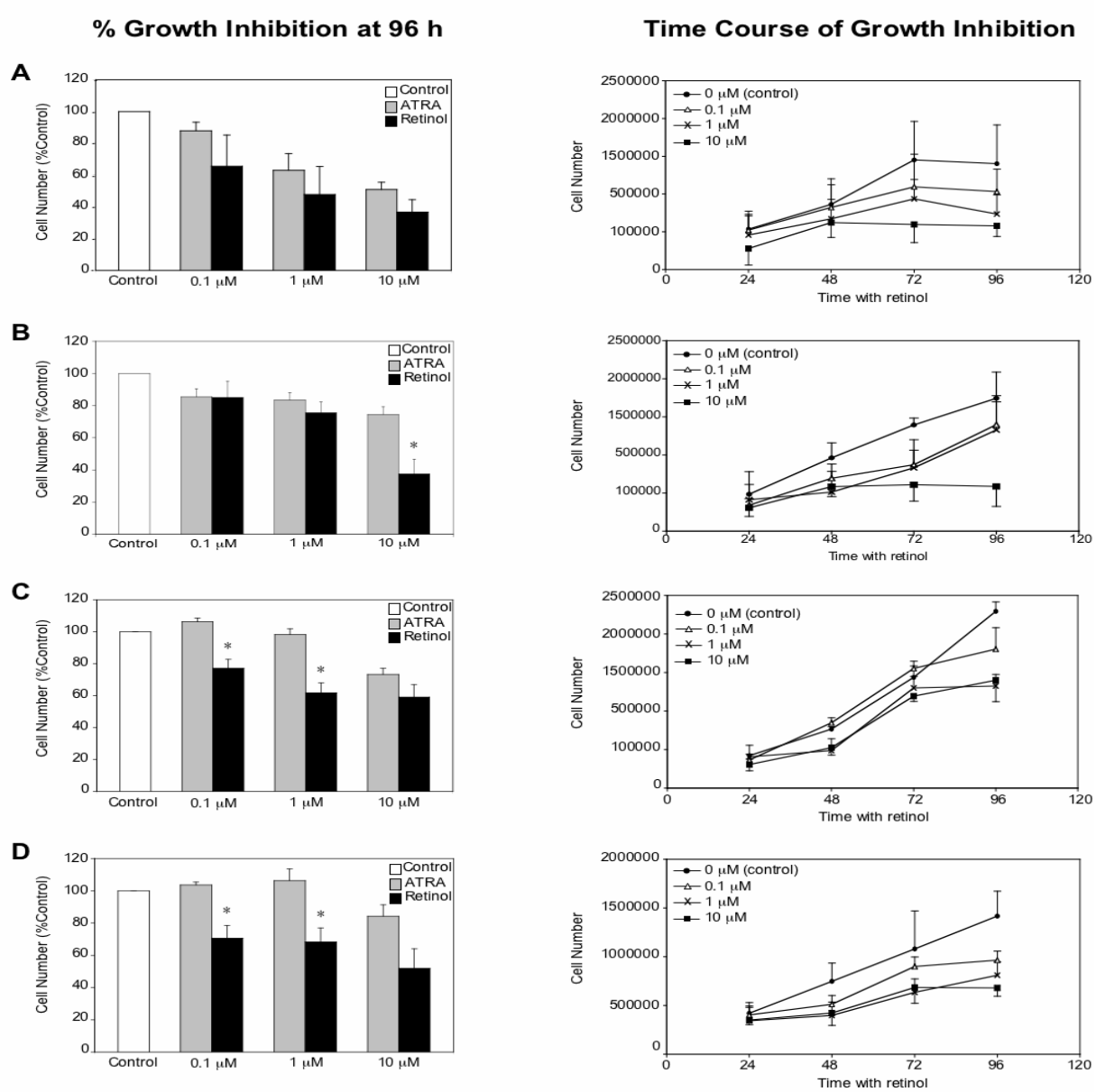

FIGURE 2.1. RETINOL INHIBITS THE GROWTH OF ATRA-RESISTANT HUMAN COLON CANCER CELLS

FIGURE 2.1. RETINOL INHIBITS THE GROWTH OF ATRA-RESISTANT HUMAN COLON CANCER CELLS. HCT-15 (A) HCT-116 (B), SW620 (C), and WiDr (D) cells were seeded and treated with 0, 0.1, 1 or 10 μM ATRA or retinol. All treatments were performed in duplicate. Cells were counted via hemocytometer daily for four days. Results represent the mean ± SE for three experiments. Panels in the left column display the percent growth inhibition exhibited by human colon cancer cell lines after 96 h of treatment with increasing amounts of ATRA or retinol. Statistical analysis was performed using t-tests comparing ATRA to retinol for each concentration. *Indicates significantly different from ATRA, $P < 0.05$. Panels in the right column show the growth rates of HCT-15 (A), HCT-116 (B), SW620 (C) and WiDr (D) cells grown for four days with increasing amounts of retinol.

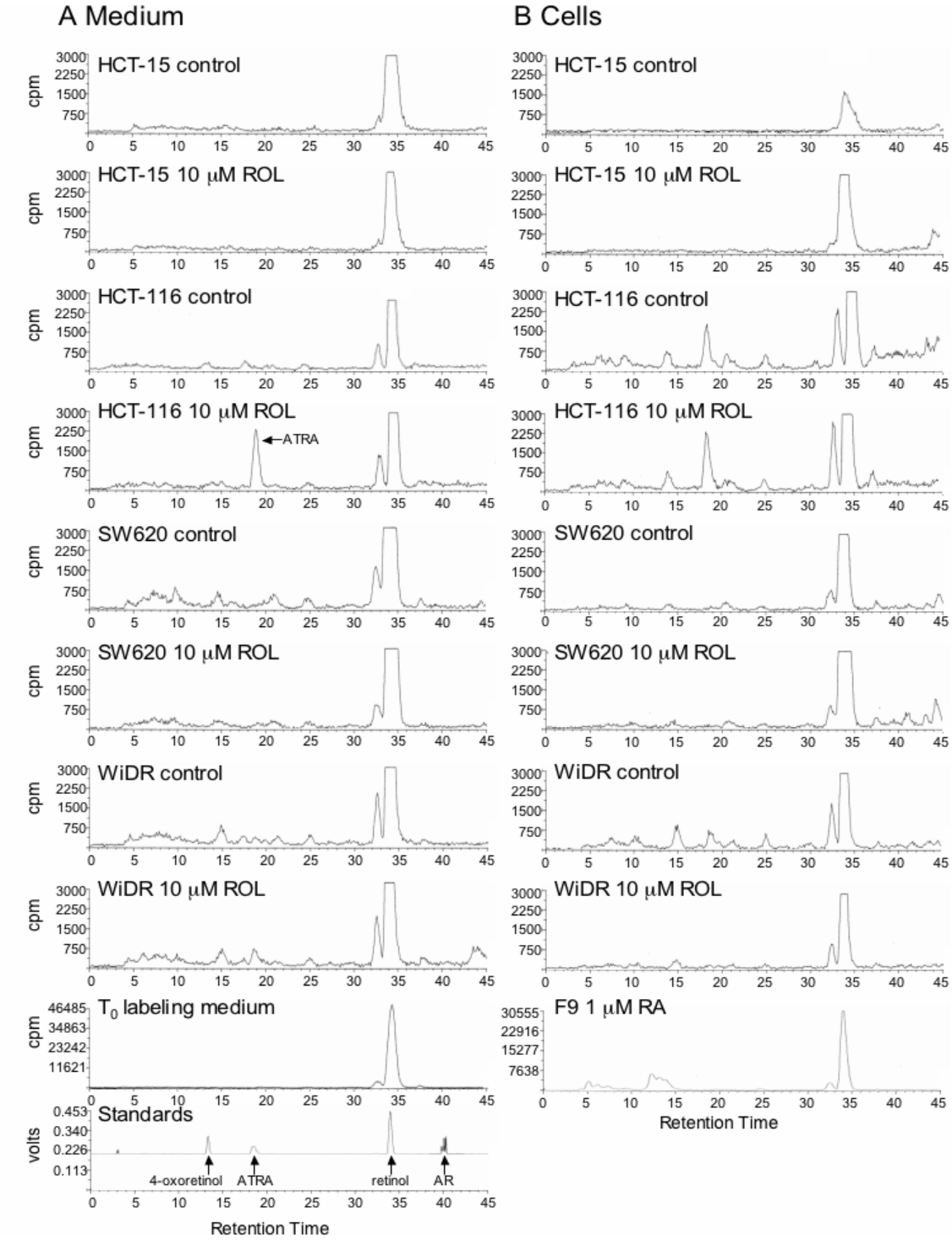

FIGURE 2.2. RETINOL IS NOT METABOLIZED TO BIOACTIVE COMPOUNDS IN HCT-15 AND SW620 CELLS BUT IS CONVERTED TO ATRA BY HCT-116 AND WIDR CELLS.

FIGURE 2.2. RETINOL IS NOT METABOLIZED TO BIOACTIVE COMPOUNDS IN HCT-15 AND SW620 CELLS BUT IS CONVERTED TO ATRA BY HCT-116 AND WIDR CELLS. Cells were plated and allowed to attach for 24 hr before addition of medium containing 0, 1, or 10 μM retinol. Cells were allowed to grow for 24, 48, 72 or 96 hr before the medium was removed and replaced with new medium containing 5% FCS and 50 nM [^{3}H]-retinol. The concentration of nonradioactive retinol in 5% FCS was ≈ 50 nM (51). Cells were allowed to incubate for another 2, 4, 8 or 16 hr before harvest. The retinoids were extracted from cell and medium samples and separated by HPLC as described (51). Data shown represent radiolabeled retinoids extracted from medium (A) or cells (B) of the indicated cell lines 48 h after treatment with 0 or 10 μmol/L retinol followed by incubation for 8 h with 50 nM [^{3}H]-retinol. The identities of the retinoids were determined by coelution with known nonradiolabeled (cold) standards included in the samples. Changes in absorbance are recorded as changes in voltage by the FloOne software that generated these chromatographs and controls the liquid scintillation counter. Thus, the units for the y-axis of "Standards" panel are volts. The slight difference in elution time between the cold standards and the [^{3}H]-peaks is due to the transit time from the photo diode array to the scintillation counter. [^{3}H]-Retinol extracted from labeling medium at time zero is shown in the left column, one panel from the bottom. F9 murine teratocarcimona cells were treated with 1 μmol/L RA for 48 hr followed by incubation with [^{3}H]-retinol for 16 hr prior to retinoid extraction. The retinoids extracted from F9 murine teratocarcimona cells are included as a positive control for 4-oxoretinol production and are shown in the last panel of the right column. Retinoid standards are shown in the bottom panel of the left column and eluted as follows: 4-oxoretinol, 13.5 min; ATRA, 18.5 min; all-*trans* retinol, 34.0 min, and AR, 39.8 to 40.5 min. This experiment was repeated twice with similar results.

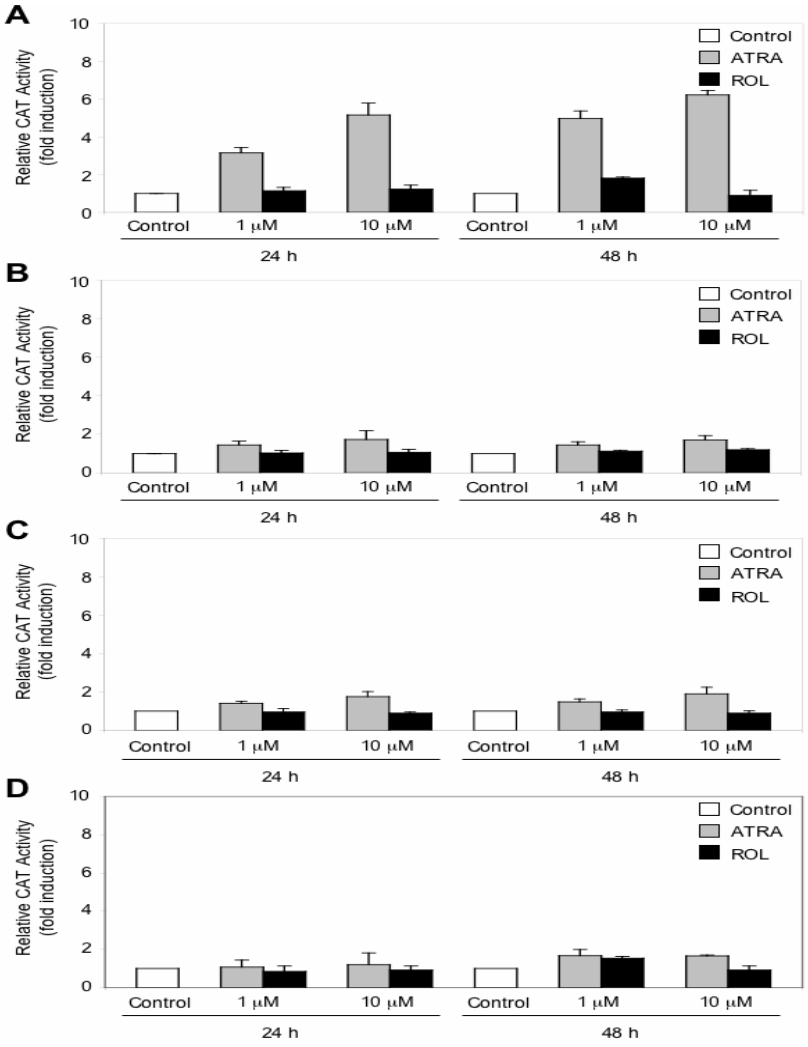

FIGURE 2.3. RETINOL DOES NOT INDUCE RARE-CAT REPORTER GENE EXPRESSION. HCT-15 (A), HCT-116 (B), SW620 (C), and WiDr (D) cells were transiently transfected with 1 μg pRARE-CAT and 0.5 μg pSV-β-gal using lipofectamine 2000. Twenty-four hours following transfection, cells were treated with 0, 1, and 10 μM RA or retinol. Cells were harvested 24 and 48 h after treatment and CAT and β-galactosidase assays were performed. CAT activity was normalized for transfection efficiency using the β-galactosidase activity. The CAT activity in control cells treated with the ethanol vehicle was set equal to one and all other values are expressed as fold induction. Values shown are the mean ± SE of three separate experiments.

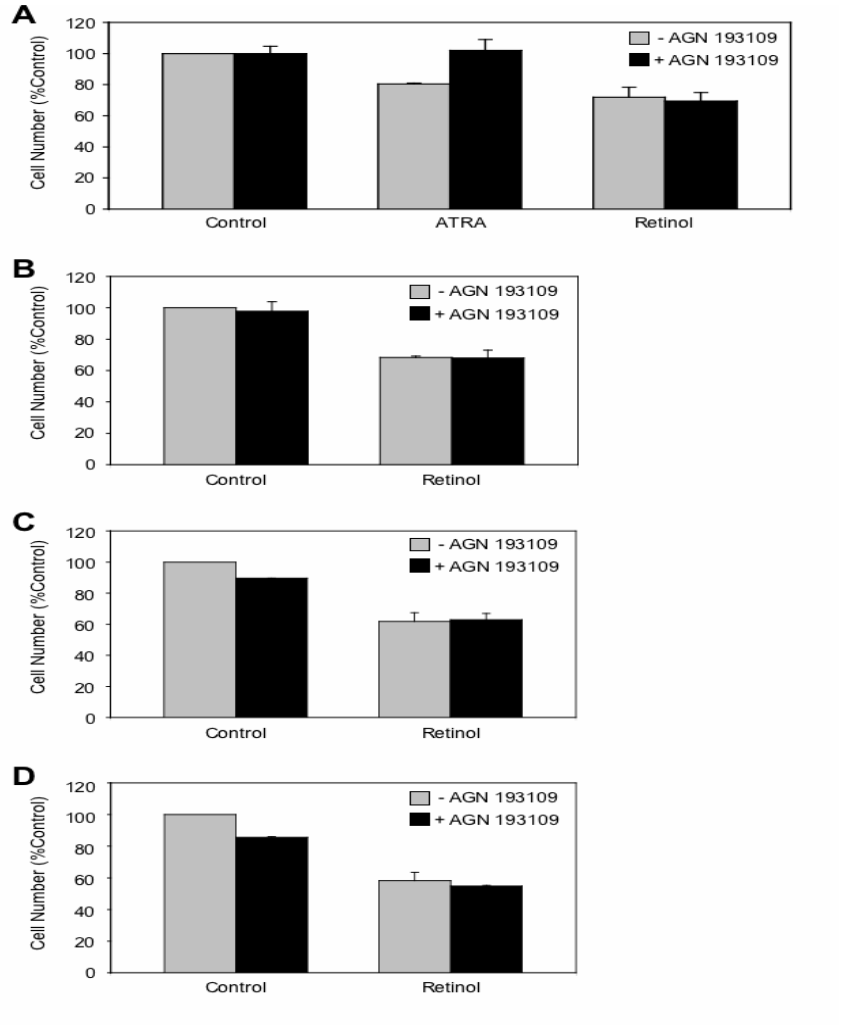

FIGURE 2.4. RETINOL DOES NOT ACT THROUGH RAR TO INHIBIT CELL GROWTH. HCT-15 (A) cells were plated with 0 or 1 μM RA or retinol with or without 10 μmol/L of the RAR pan-antagonist, AGN 193109, for 48 h. HCT-116 (B), SW620 (C), and WiDr (D) were treated with 0 or 1 μM retinol with or without 10 μM AGN 193109 for 96 h. All treatments were plated in duplicate. Data shown are the mean of two separate experiments ± SE.

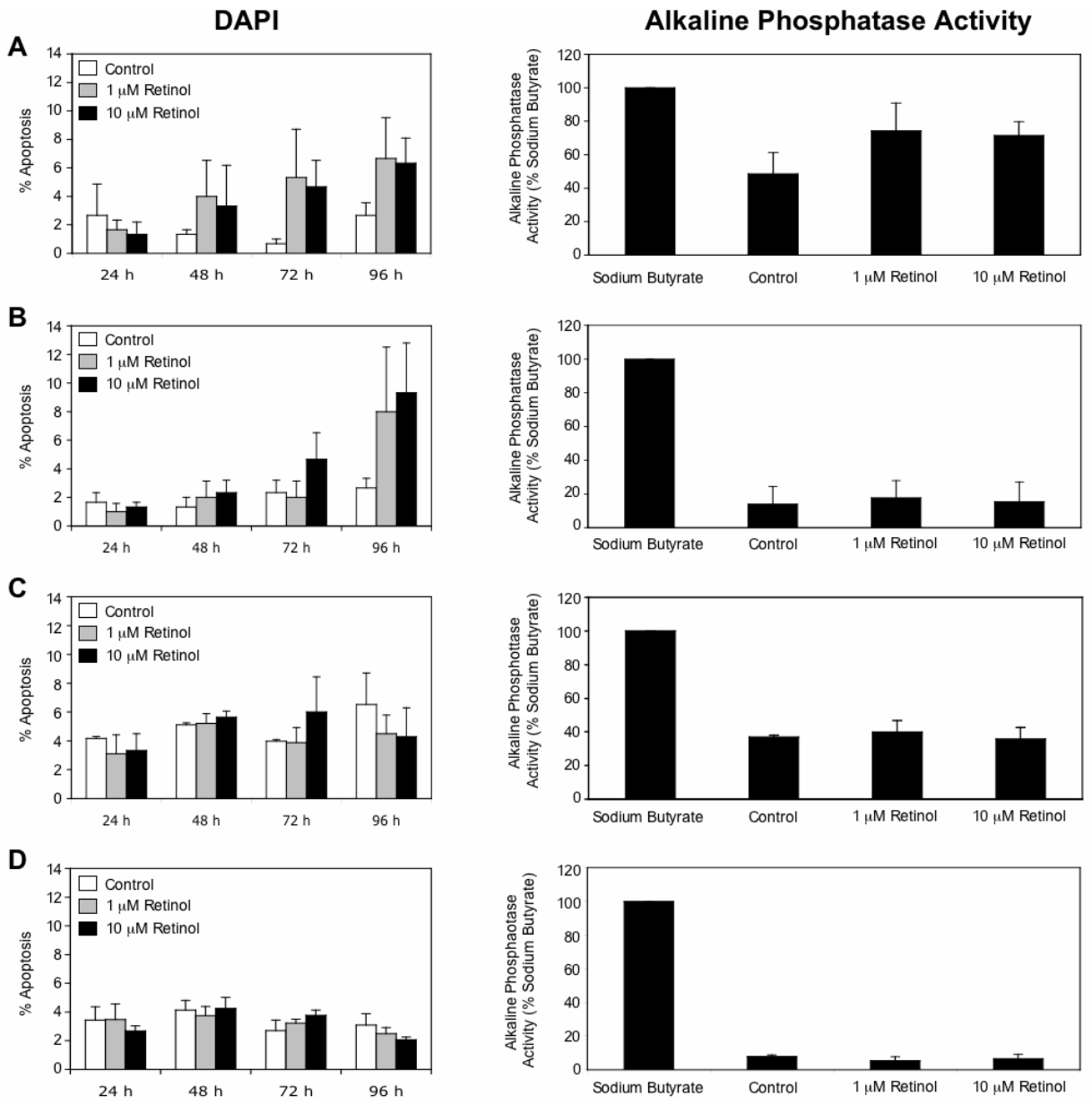

FIGURE 2.5. RETINOL DOES NOT INDUCE APOPTOSIS OR CELLULAR DIFFERENTIATION IN ATRA-RESISTANT HUMAN COLON CANCER CELL LINES.

FIGURE 2.5. RETINOL DOES NOT INDUCE APOPTOSIS OR CELLULAR DIFFERENTIATION IN ATRA-RESISTANT HUMAN COLON CANCER CELL LINES. HCT-15 (A), HCT-116 (B), SW620 (C), and WiDr (D) cells were plated and treated with 0, 1 or 10 μM retinol as described in Materials and Methods. To measure apoptosis (left column), floating and adherent cells were harvested every 24 hr, centrifuged and washed with PBS to remove all traces of media. Cells were stained with 2 μg/mL DAPI for 10 min in 37ºC before observation. To measure cellular differentiation (right column), cells were plated as described and treated with 0, 1, or 10 μM retinol or 2 mM sodium butyrate for 96 h. Alkaline phosphatase activity was determined as described (Turowski) by the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol at 410 nm. Alkaline phosphatase enzyme activity is expressed as percent positive control. Data shown are the mean ± SE for three independent experiments.

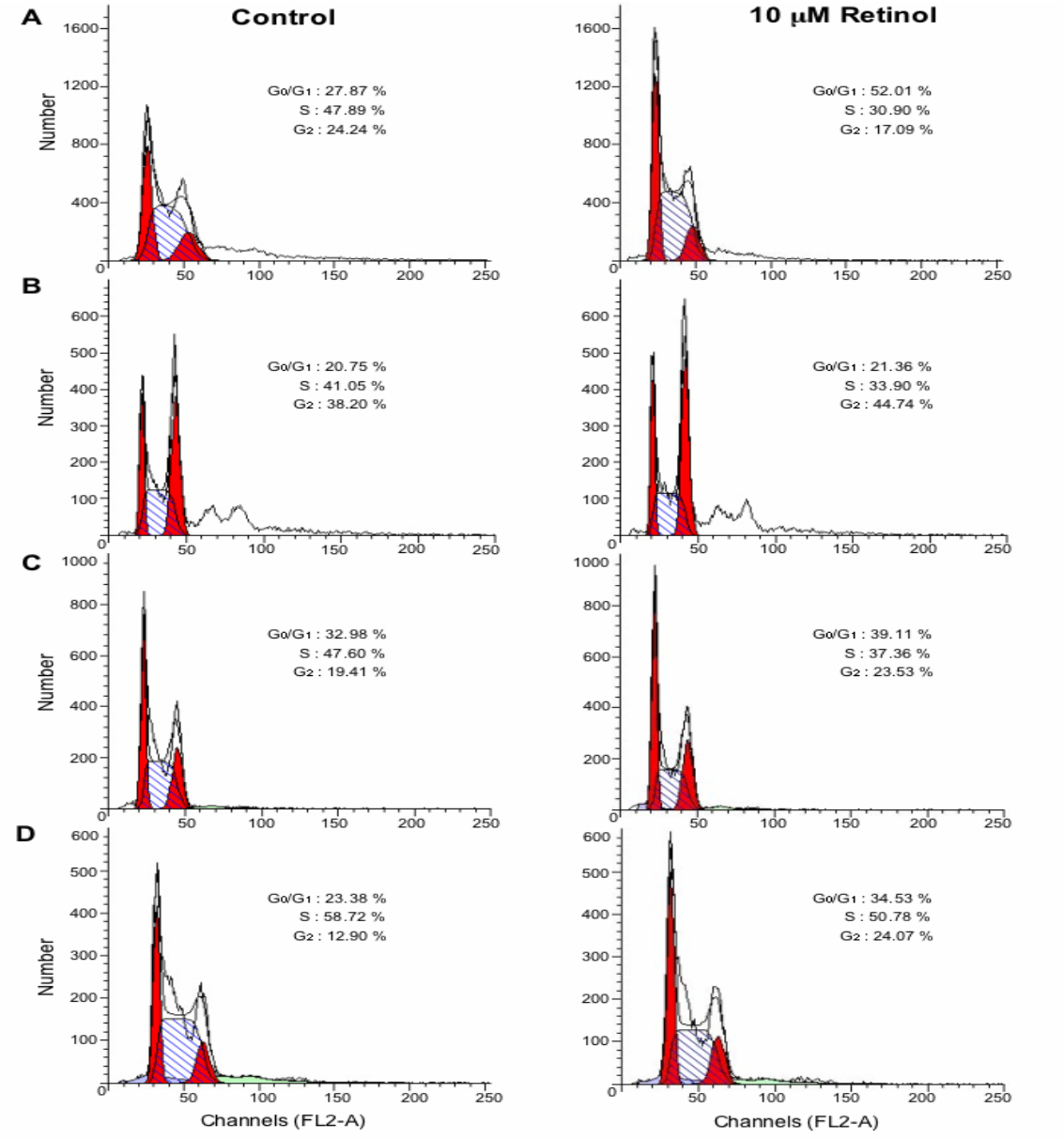

FIGURE 2.6. RETINOL ALTERS CELL CYCLE PROGRESSION BUT DOES NOT INDUCE APOPTOSIS.

FIGURE 2.6. RETINOL ALTERS CELL CYCLE PROGRESSION BUT DOES NOT INDUCE APOPTOSIS. HCT-15 (A), HCT-116 (B), SW620 (C), and WiDr (D) cells were synchronized with serum starvation for a total of 48 h and the addition of aphidicholin during the last 24 h of serum starvation. Following synchronization, fresh medium containing 10 % FCS and 0 (left column) or 10 μM retinol (right column) was added for 24 h, prior to fixation and staining with propidium iodide. At least 10,000 cells were analyzed per sample using a FACS Calibur machine. Absence of a sub-$G_{0/1}$ indicates that apoptosis was not induced by retinol treatment. Dark gray, $G_{0/1}$ and $G_{2/M}$; hatched, S-phase. One representative experiment of two is shown.

Chapter 3: Retinol inhibits the invasion of retinoic acid-resistant colon cancer cells *in vitro* and decreases matrix metalloproteinase mRNA, protein, and activity levels

ABSTRACT

Retinol inhibits the growth of *all-trans*-retinoic acid (ATRA)-resistant human colon cancer cell lines through a retinoic acid receptor (RAR)-independent mechanism. The objectives of the current study were to determine if retinol inhibited the invasion of *all-trans*-retinoic acid (ATRA) -resistant colon cancer cells independent of RAR and the effects of retinol on matrixmetalloproteinases (MMP). Retinol inhibited the migration and invasion of two ATRA-resistant colon cancer cell lines, HCT-116 and SW620, in a dose-dependent manner. To determine if transcription, particularly RAR-mediated transcription, or translation of new genes was required for retinol to inhibit cell invasion, cells were treated with retinol and cycloheximide, actinomycin D, or a RAR pan-antagonist. Treatment of cells with retinol and cycloheximide, actinomycin D, or a RAR pan-antagonist did not block the ability of retinol to inhibit cell invasion. In addition, retinol decreased MMP-1 mRNA levels in both cell lines, MMP-2 mRNA levels in the SW620 and MMP-7 and -9 mRNA levels in the HCT-116 cell line. Retinol also decreased the activity of MMP-2 and -9 and MMP-9 protein levels while increasing TIMP (tissue inhibitor of matrixmetalloproteinase)-1 media levels. In conclusion, retinol reduces the metastatic potential of ATRA-resistant colon cancer cells via a novel

RAR-independent mechanism that may involve decreased MMP mRNA levels and activity.

INTRODUCTION

Colorectal cancer is the third most common cancer and cause of death due to cancer in the United States (1). Death is generally not due to the primary, localized tumor, but to the metastasis of the cancer to other tissues, primarily the liver. The escape of primary tumor cells into the circulation (intravasation) and the invasion of these tumor cells into the new target tissue (extravasation) to establish metastases requires digestion of and migration through the extracellular matrix (ECM). The digestion of the ECM is performed by matrixmetalloproteinases (MMP), a large family of proteases [For a review please see: (8,9)]. MMP are classified by their substrates into different groups, collagenases, gelatinases, stromelysins, membrane-type MMP, matrilysins, and macrophage elastases, etc. Increased serum or tissue levels and activity of MMP-1, -2, -7, -9 and -13 are associated with colorectal cancer progression [(83,84); for a review please see: (9)]. MMP must be cleaved to be active and MMP activity is regulated both at cleavage and total protein levels. MMP activity is also regulated by TIMPs (tissue inhibitor of matrixmetalloproteinase), which function as endogenous protease inhibitors [For a review please see: (9,85)]. TIMP-1 inhibits the activity of all MMP. In addition, TIMP-1 binds to pro-MMP-9 to inhibit its conversion to active MMP-9. [For a review please see (9)].

The retinoids, a group of compounds consisting of vitamin A (retinol), its natural metabolites, and several synthetic compounds, have been shown to inhibit metastasis in a variety of model systems. For example, dietary retinyl palmitate supplementation decreases malignant melanoma metastasis in mice (86). In addition, *all-trans*-retinoic acid (ATRA), an active metabolite of dietary vitamin A, decreases gastric cancer (87) and colon cancer cell invasion (88) *in vivo*. Numerous studies have shown that ATRA reduces tumor cell invasion and metastasis *in vitro* by decreasing MMP mRNA, protein levels or enzyme activity as well as increasing TIMP mRNA or protein levels. However, to our knowledge, the ability of retinol, the form of vitamin A derived from the diet, to inhibit tumor cell invasion by altering MMP mRNA and protein levels or activity has not been explored.

ATRA exerts its effects by binding to retinoic acid receptors (RARs). The RARs function as heterodimers with retinoic X receptors (RXRs). When ligand bound, the RAR/RXR heterodimer activates gene transcription via retinoic acid response elements (RAREs) located in the regulatory regions of retinoid-responsive genes [for a review please see (89)]. The clinical effectiveness of ATRA is limited by ATRA-resistance, defined as the inability of ATRA to inhibit cell growth and induce differentiation. ATRA-resistance is a common phenomenon associated with cancer progression and is believed to be due to the inability of ATRA to induce RAR gene expression in ATRA-resistant cells (90-93).

Because the diet contains almost no ATRA, the use of exogenous ATRA to study the effects of vitamin A assumes that all of the biological phenomena attributed to retinol are due to ATRA. Preformed dietary vitamin A is consumed as retinyl esters that are

cleaved within the intestinal lumen to yield retinol. The concentration of ATRA in the circulation is very low (1-14 nM) (94). In contrast, serum retinol concentrations range from 0.5 to 2 μM (55). Therefore, colonocytes are primarily exposed to retinol, the focus of the present study, via the diet and the circulation. We have previously shown that retinol, but not ATRA, inhibits the growth of ATRA-resistant colon cancer cell lines through an ATRA and RAR-independent mechanism (95). The use of ATRA-resistant cell lines allows us to examine the effects of retinol exclusive of those of ATRA. For example, the ATRA resistant cell lines used in this study lack one or more of the RAR and either lack the ability to metabolize retinol to ATRA, as is the case for the SW620 cell line, or produce only very small amounts of ATRA from retinol, as is the case for the HCT-116 cell line (95). Importantly, treatment of these cells lines with ATRA or retinol does not induce the transcription of a RARE-chloramphenicol acetyltransferase (CAT) reporter construct in either cell line and the RAR pan-antagonist, AGN 193109, was unable to block the inhibitory effects of retinol on tumor cell growth (95). Inhibition of primary tumor growth is only one aspect of a successful chemotherapeutic agent, therefore the objective of the present study was to examine the ability of retinol to inhibit ATRA-resistant colon cancer cell metastasis *in vitro.* Because ATRA has been shown to inhibit cell invasion by decreasing MMP activity and increasing TIMP, we also explore the effect of retinol on MMP mRNA and protein levels, MMP enzyme activity, and TIMP protein concentration.

MATERIALS AND METHODS

Tissue Culture

The human carcinoma cell line, HCT-116, and the human colorectal adenoma cell line, SW620, were obtained from the American Type Culture Collection (Manassas, VA) and grown as recommended. HCT-116 cells were grown in McCoy's medium and SW620 cells in DMEM in a humidified atmosphere at 37°C with 5% CO_2. All medium was supplemented with 10% FBS (fetal bovine serum) and antibiotics (1000 U/mL penicillin and 1000 μg/mL streptomycin).

Migration and Invasion Assays

HCT-116 and SW620 cells were serum starved for 48 h before seeding at a density of 1 x 10^5 cells/well. Uncoated Boyden chambers were used to assess the effect of retinol on cell migration while Matrigel coated Boyden chambers (Becton Dickinson, Franklin Lakes, NJ) were used to determine the ability of retinol to inhibit cell invasion through a basement membrane. To measure cell migration, the upper portion of the chambers contained 0 (ethanol vehicle control), 0.1, 1, or 10 μM retinol. Retinol was prepared as 10 mM stock in 100 % ethanol. All treatments, including control, received equal volumes of ethanol vehicle and all retinol manipulations were performed in the dark. A filter containing pores 8 μm in diameter separated the cells from a lower chamber containing 10% FBS, which served as a chemoattractant. After 8 h all cells were removed from the upper chamber using a cotton swab. Cells that had migrated

through the membrane were fixed in methanol prior to staining with propidium iodide. The bottom of the filter was examined microscopically and the number of stained cells present in ten random fields of view were counted with a 20X objective on an Olympus upright fluorescence microscope to determine cell migration as described (96).

To examine the effect of retinol on cell invasion, the upper portion of the chambers contained 0 (ethanol vehicle control), 1, or 10 μM retinol while the lower portion, separated by an 8 μm pore-sized filter coated with Matrigel, contained 20 ng/ml hepatic growth factor (HGF) (Sigma-Aldrich, St. Louis, MO) and 10% FBS, which served as chemoattractants. Cell invasion was measured after 24 h. All cells remaining in the upper portion of the chamber were removed. Cells that had migrated through the membrane were fixed in methanol prior to staining with propidium iodide and quantified, as described above.

To determine if the effects of retinol on cell invasion required the transcription, particularly RAR-mediated transcription, or translation of new genes, cells were treated with 2 mg/ml actinomycin D, 10 μg/ml cycloheximide, or 10 μM of a RAR pan-antagonist (AGN193109, Allergan, Irvine, Ca), with or without 1 or 10 μM retinol added to the upper portion of Matrigel-coated Boyden chambers. Twenty-four h later, cell invasion was quantitated as described above.

To confirm the role of MMP-2 and –9 in colon cancer invasion, MMP activity was blocked using specific antibodies against MMP-2 (#AB809, Chemicon, Temecula, CA) and MMP–9 (#AB19016, Chemicon, Temecula, CA). Control cells were incubated with a non-specific IgG antibody (#SC-2027, Santa Cruz Biotechnology, Santa Cruz, CA). HCT-116 and SW620 cells were plated in Matrigel-coated Boyden chambers as

described above. Antibody (10 μg/ml) was added to the upper portion of the chambers and 20 ng/ml of HGF was added to the lower portion of the chamber, containing medium supplemented with 10% FCS, to serve as a chemo-attractant. After 24 h, cells that had moved through the membrane were visualized by propidium iodide staining and quantitated.

RNA extraction and quantitative real time RT-PCR

Cells were plated at a density of 1x 10^6 cells/100 mm dish. After 24 h, cells were treated with 0 (ethanol vehicle control), 1, or 10 μM retinol. Twenty-four h later total RNA was extracted with RNAstat (Tel-Test, Inc, Friendswood, Texas). Reverse transcription was performed from 2 μg of total RNA using oligo-dT and AMV reverse transcriptase (Promega, Madison, WI) according to manufacture's instructions. The RNA was incubated for 10 min at 70°C and subsequently subjected to reverse transcription for 15 min at 42°C, followed by heating at 95°C for 5 min. The sequences of the primers used are displayed in Table 1. The identity of the amplified PCR product was confirmed by sequence analysis. Quantitative real time-reverse transcriptase (RT)-PCR was performed with SYBR Green dye (Perkin-Elmer-Applied Biosystems, Foster City, CA) using an ABI 7900HT (Perkin-Elmer-Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Briefly, an initial denaturation step (10 min at 95°C) was followed by a two-step PCR (15 s at 95°C; 1 min at 60°C, 40 cycles). PCR reactions were performed in duplicate. Relative amounts of MMP cDNA were

calculated by the comparative CT method (97). CT values obtained for the different MMP were normalized to corresponding CT values of GAPDH.

Zymography

HCT-116 and SW620 cells were plated at a density of 2 x 10^6 cells/100 mm dish. Cells were washed with PBS twice and treated with 0 (ethanol vehicle control), 1, or 10 μM retinol in serum free media for 24 h. MMP-2 activity was detected in serum-free media concentrated using a centricon column with a 10 kDa cutoff value (Millipore, Volketswil, Switzerland). MMP-9 activity was measured in serum-free media incubated with MMP-9 antibody at a 1:500 dilution and 20 μl of protein A/G plus agarose bead/mL (#SC-2003, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The following day the beads were washed with 1% NP-40 three times and then once with PBS. Sample buffer (0.1 M Tris·HCl (pH 8.6), 10% Glycerol, 0.0025% bromophenol blue) was used to elute MMP-9 from the agarose beads prior to zymography.

Zymograms were prepared as described previously (98). Equal amounts of protein were separated using a 0.1% gelatin/10% SDS-PAGE gel. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 1 h at room temperature and incubated overnight with developing buffer (Invitrogen, Carlsbad, CA) at 37°C. Gels were stained with 0.25 % Coomassie Blue Dye R250 and distained with 30% methanol and 10% acetic acid.

Western blot analysis

To determine TIMP-1 and MMP-9 protein levels, HCT-116 and SW620 cells were plated and treated with retinol as described above for zymography. Following treatment, the serum-free media was concentrated 10-fold using centricon columns with a 10 kDa cutoff value (Millipore, Volketswil, Switzerland). The concentrated media were electrophoresed through 8% SDS-PAGE gels for MMP-9 and 12 % SDS-PAGE gels for TIMP-1 and transferred to nitrocellulose membranes. To examine the level of intracellular TIMP-1, HCT-116 and SW620 cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, 50 mM Tris, 1 mg/ml leupeptin, 1 mM DTT, 2 mM $NaOV_4$, 1 mg/ml PMSF, 1 mg/ml trypsin inhibitor, and 10 mM aprotinin) 24 h after treatment with retinol. Cell lysate protein concentrations were determined using the BioRad DC protein assay kit (Hercules, CA). Protein (50 μg) was electrophoresed through a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. Membranes were blocked with 5% milk in TBST (10 mM Tris, pH 8, 150 mM NaCl, and 0.5% Tween-20) for 1 h at room temperature before MMP-9, TIMP-1, and β-actin antibodies (Sigma-Aldrich, #A2066, St. Louis, MO) were added at a 1:2000 dilution. MMP-9 (#AB19016), and TIMP-1 (#AB800-50) antibodies were purchased from Chemicon (Temecula, CA). After incubation with the corresponding secondary antibody at a dilution of 1:2000, immunoreactivity was detected using the Pierce Horseradish Peroxidase Super Signal West Pico Chemiluminescent Substrate kit (Rockford, IL).

Statistical Analysis

Values shown are the mean ± SEM of at least three independent experiments unless otherwise indicated. All statistical tests were performed using SPSS (Apache Software Foundation, Wilmington, DE, version 12.0 for Windows). Data were analyzed using two-tailed t-tests comparing each retinol concentration to control (0 μM) with the exception of the data shown in Fig. 2 and 5. In the case of Fig. 2, two-tailed t-tests were used to compare samples with and without cycloheximide, actinomycin D, and AGN 193109. In Fig. 5, two-tailed t-tests were used to compare cell invasion between each specific MMP antibody and control cells treated with a non-specific IgG antibody. Results were considered significantly different at $P < 0.05$.

RESULTS

Retinol inhibits cell migration and cell invasion in vitro

The ability of retinol to inhibit the migration of ATRA-resistant colon cancer cells was examined in two ATRA-resistant human colon cancer cell lines: HCT-116 (90) and SW620 (99). Serum concentrations of retinol range from 0.5 to 2 μM (55). Therefore, 0.1 μM was selected to represent a sub-physiological, and 1 μM a physiological, concentration of retinol. The highest level, 10 μM retinol, was used as a pharmacological, but potentially therapeutically relevant, concentration. In HCT-116 cells, 0.1, 1, and 10 μM retinol decreased cell migration to 50.0 ± 17.1%, 35.4 ± 15.2%, and 27.9 ± 17.1% of vehicle control, respectively (Fig. 3.1A). Treatment with 0.1, 1, and

10 μM retinol reduced SW620 cell migration to 58.7 ± 11.6%, 36.5 ± 8.9%, and 36.4 ± 7.2% of vehicle control, respectively (Fig. 3.1A). Treatment with retinol for 8 h did not decrease cell number (data not shown).

Because cell migration is only one facet of metastatic potential, we determined whether retinol could inhibit the ability of colon cancer cells to digest and move through Matrigel, a basement membrane-like protein matrix, *in vitro*. The ability of HCT-116 cells to invade through Matrigel was decreased to 52.6 ± 15.9% and 40.0 ± 6.7% of vehicle control by 1 and 10 μM retinol, respectively (Fig. 3.1B). SW620 cell invasion was decreased by 1 and 10 μM retinol to 81.2 ± 19.0% and 46.0 ± 15.0% of vehicle control, respectively (Fig. 3.1B). Cell number was not affected after 24 h of treatment with retinol (data not shown). These data show that retinol inhibits both cell motility and invasion through an ECM.

The effect of retinol on cell invasion is RAR-independent

To elucidate whether retinol mediated cell invasion through increased gene transcription or mRNA translation, HCT-116 and SW620 cells were treated with cycloheximide or actinomycin D. HCT-116 cells treated with 1 μM retinol and actinomycin D were significantly less invasive than cells treated with 1 μM retinol alone, indicating that transcription may be required for invasion in this cell line (Fig. 3.2B). Importantly, treatment with cycloheximide (Fig. 3.2A) or actinomycin D (Fig. 3.2B) did not block the inhibitory effect of retinol on cell invasion in either cell line, suggesting that

increased gene transcription and translation do not mediate the inhibitory effect of retinol in cell invasion.

In most ATRA-sensitive cells, the actions of retinol are mediated by its metabolite, ATRA, which acts by binding to RARs and inducing gene expression via RAREs. However, HCT-116 cells lack all RAR (90) and, while SW620 cells may express some RARs (93,99), we have shown that retinol inhibits HCT-116 and SW620 colon cancer cell growth independent of ATRA and the RARs (95). To determine if the inhibitory effect of retinol on cell invasion was also RAR-independent and to confirm our actinomycin D data, we used the RAR pan-antagonist, AGN193109, to block RAR signaling and ensure that any RARs present would be inactive. This antagonist, when added at 10 times the concentration of agonist, blocks the ability of agonist to bind to RAR (100). Cells were treated with 0 or 1 μM retinol with and without 10 μM AGN 193109. The 10 μM concentration of retinol was not examined in this experiment because 100 μM AGN193109 was toxic to the cells. As can be seen in Fig. 3.2C, the RAR-antagonist did not alleviate the inhibitory effect of retinol on cell invasion. These data show that retinol inhibits the invasion of colon cancer cells independent of RAR and new protein or mRNA synthesis.

Retinol decreases MMP mRNA levels

MMP facilitate the cell invasion by digesting the ECM. To determine whether the retinol-induced decrease in cell invasion was due solely to lower cell motility or also to a decrease in ECM digestion, we examined the mRNA levels of five MMP using

quantitative real-time RT-PCR. The gelatinases MMP-2 and MMP-9, matrilysin MMP-7, and the collagenases MMP-1 and MMP-13 are believed to be important in colorectal cancer metastasis (8). Treatment with 10 μM retinol reduced MMP-2 mRNA levels to 63 ± 8% of control in SW620 cells but not in HCT-116 cells (Fig. 3.3A). In contrast, MMP-9 mRNA levels were reduced to 69 ± 4% of control by treatment with 10 μM retinol in HCT-116 cells (Fig. 3.3B). Treatment with 1 μM retinol reduced MMP-9 levels to 57 ± 1.5 % of control in the SW620 cell line but treatment with 10 μM retinol did not lower MMP-9 mRNA levels, indicating that different concentrations of retinol may exert varying effects on MMP-9 mRNA levels in this cell line. The mRNA level of MMP-7 was decreased in HCT-116 cells to 60 ± 11% of vehicle control by 10 μM retinol treatment, but MMP-7 mRNA was not affected by retinol treatment in SW620 cells (Fig. 3.3C). Treatment with 10 μM retinol decreased MMP-1 mRNA levels to 48 ± 8% and 44 ± 8% of vehicle control in HCT-116 and SW620 cells, respectively (Figure 3.3D). In contrast, retinol treatment of HCT-116 and SW620 cells did not change mRNA levels of MMP-13 (Fig. 3.3E). These data suggest that retinol may decrease cell invasion by reducing MMP mRNA levels *in vitro*.

Retinol decreases gelatinase (MMP-2 and -9) enzyme activity

We further investigated whether retinol decreased the enzyme activity and protein levels of the MMP whose transcription it altered. Retinol treatment decreased MMP-9 enzyme activity and total levels of active MMP-9 in the conditioned media obtained from both the HCT-116 and SW620 cell lines (Fig. 3.4). No pro-MMP-9 was detected in the

conditioned media (Fig. 3.4B and D). Retinol treatment also decreased MMP-2 activity in conditioned media from SW620 cells (Fig. 3.4C) but did not affect pro-MMP-2 levels in this cell line. Although MMP-2 mRNA was decreased by retinol treatment in HCT-116 cells (Fig. 3.3), neither pro- nor active-MMP-2 levels were decreased by treatment with retinol in conditioned media from HCT-116 cells (Fig. 3.4A). Although retinol decreased the mRNA levels of MMP-1 and -7 and these enzymes are important for cell invasion, we were unable to detect MMP-1 and -7 enzyme activity or protein using the MMP-1 Biotrak Activity Assay System (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), which detects both active and total MMP-1, or casein zymogel and western blotting of conditioned media, to detect MMP-7, in both cell lines (data not shown) potentially because these cell lines secrete low levels of MMP-1 and -7 (101,102). These data suggest that retinol may reduce the invasion of colon cancer cell lines *in vitro* by reducing the protein levels and activity of the gelatinases, MMP-2 and, particularly, MMP –9.

MMP –2 and –9 are involved in colon cancer cell invasion

Next, we verified that the MMP whose protein or activity levels were decreased by retinol treatment played a role in the invasion of these cell lines through Matrigel. Specific, neutralizing antibodies were used to block the activity of MMP-2 and -9 in *in vitro* invasion assays. Cell invasion was decreased to 19 ± 12% by an MMP-9 neutralizing antibody in the HCT-116 cell line (Fig. 3.5A). However, in the HCT-116 cell line, the MMP-2 neutralizing antibody only decreased cell invasion to 59 ± 10% of

that exhibited by control cells, indicating that MMP-2 may play a smaller role in the invasion of this cell line through Matrigel than MMP-9. This data reflects that shown in Fig. 3.4A where MMP-2 activity was not decreased, but MMP-9 activity was decreased, by retinol treatment in HCT-116 cells. In the SW620 cell line, invasion was decreased to 26 ± 7% and 29 ± 27% by MMP-2 -and -9 neutralizing antibodies, respectively (Fig. 3.5B), suggesting that MMP-2 and –9 are involved in SW620 colon cancer cell invasion through Matrigel. Similarly, both MMP-2 and –9 activity were decreased by retinol treatment in SW620 cells (Fig. 3.4B).

Retinol increases TIMP-1 protein levels in conditioned media

Because MMP-9 activity is regulated by TIMP-1, western blot analysis of cell lysates and conditioned medium as well as quantitative real-time RT-PCR were used to examine the effect of retinol on TIMP-1 protein and mRNA levels. Retinol treatment increased TIMP-1 protein levels in media but not in cell lysates (Fig. 3.6A and B). Unlike other TIMPs, TIMP-1 is inducible. TIMP-1 protein concentration is controlled at the level of transcription, mRNA stability, protein degradation, and endocytosis (85). Retinol slightly decreased TIMP-1 mRNA levels to 86 ± 5% and 85 ± 4% of control in HCT-116 cells treated with 1 and 10 μM retinol, respectively, but not in SW620 cells (Fig. 3.6C-D). Importantly, retinol does not increase TIMP-1 mRNA or protein levels in the cell lysates (Fig. 3.6C-D) and the inhibitory effect of retinol on cell invasion is not blocked by actinomycin D or cycloheximide treatment (Fig. 3.2). Therefore, if TIMP-1

is mediating the inhibitory effect of retinol on cell invasion, retinol may be increasing extracellular TIMP-1 levels by decreasing TIMP-1 degradation or endocytosis.

DISCUSSION

This study demonstrates that retinol decreases the invasion of human colon cancer cell lines *in vitro* (Fig. 3.1). The inhibitory effects of retinol are not dependent upon increased gene transcription via RAR or mRNA translation (Fig. 3.2). Depending on cell line, retinol treatment also decreased the mRNA levels of MMP-1, 2, -7 and -9 (Fig. 3.3) as well as the activity of MMP-2 and -9 and total media MMP-9 protein levels (Fig. 3.4). The specific MMP affected varied with cell line but the ability of retinol to decrease MMP-9 protein and activity levels was consistent between both cell lines. TIMP-1 binds to pro-MMP-9, inhibiting the activation of pro-MMP-9 (9). TIMP-1 levels were also increased by retinol treatment in the media collected from both cell lines (Fig. 3.6). Taken together, these data suggest that retinol, the form of vitamin A derived from the diet, inhibits the invasion of colon cancer cell lines *in vitro* by decreasing their ability to digest ECM proteins.

It is important to note that the colon cancer cell lines used in the present study produce little (HCT-116) or no (SW620) ATRA (95). Other groups have also found that colon cancer cell lines lack the ability to synthesize ATRA from retinol (103). In addition, the colon cancer cell lines used in the present study do not convert retinol to 4-oxoretinol or anhydroretinol, two naturally occurring retinoids capable of inhibiting cell growth (95). Therefore, we hypothesize that retinol itself, and not a bioactive

metabolite, inhibits colon cancer cell invasion. It is possible that an unknown and undetected bioactive metabolite of retinol was formed that existed only briefly and was not identified in our previous study, however we have also previously shown that retinol cannot induce CAT-activity in cells transfected with a pRARE-CAT construct (95). In the present study, the inability of a pan-RAR antagonist to block the inhibitory effects of retinol on cell invasion (Fig. 3.2) supports our conclusion that retinol inhibits cell invasion by an ATRA and RAR-independent mechanism.

Although the cell lines used in the present study either do not express RAR or express only some RAR (90,93,99) we used the RAR pan-antagonist, AGN 193109, to block any potential RAR activation in the current study and ensure that the effects of retinol on cell invasion would be exclusive of ATRA and RAR. AGN 193109 exhibits a high affinity for all RAR (100) and we have shown previously that AGN 193109 blocks the inhibitory effects of ATRA on ATRA-sensitive cell growth (95). Although a genetic approach would have been more specific, the dominant negative RAR construct available is activated by retinol (104), making it inappropriate for this study.

The ability of MMPs to digest the ECM is regulated at the mRNA, protein, and, most importantly, enzyme activity level. The current study is the first, to our knowledge, to show that retinol inhibits colon cancer cell invasion *in vitro* by decreasing MMP-1, -2 and –9 mRNA, MMP-9 protein, and MMP-2 and –9 activity exclusive of ATRA and RAR. *In vitro,* ATRA reduces breast cancer cell invasion by decreasing MMP-9 activity (105). ATRA has also been shown to decrease MMP-1 and -9 activity in diabetic human skin organ culture (106) and MMP-7 protein excretion and active MMP-7 levels in the human colon cancer cell line, BM314 (88). ATRA has been

shown to decrease MMP-1 mRNA levels in MDA-MB-231 cells (107) and decrease MMP-1 and –2 mRNA and protein levels while increasing TIMP-1 and 2 protein levels in cultured melanoma cells (108). Treatment of rat invasive prostate adenocarcinoma cells with ATRA decreased cell invasion by inhibiting MMP-2 and -9 activity (109). Finally, ATRA has also been shown to decrease MMP-9 and increase TIMP-1 gene expression in murine lung alveolar carcinoma (110). These studies indicate that ATRA can alter MMP mRNA, protein and enzyme activity levels as well as increase TIMP-1 concentrations in a variety of cell systems. In the studies mentioned above, the MMP affected by ATRA varied with cell type, reflecting our data. Interestingly, MMP-2 and-9 appear to be the most frequently regulated by ATRA.

Serum and tissue levels of the gelatinases MMP-2 and –9 are correlated with colon cancer stage and prognosis in animal and clinical studies [For a review please see: (9)]. Collins *et al* (10) and Ornstein and Cohn (11) showed that MMP-2 mRNA was significantly increased in Duke's stage B and C tumors. Koumura *et al* (111) found that a higher incidence of MMP-9 expression in colorectal tumors occurred when liver metastases were present. MMP-2 protein levels also increase as polyps progress to adenocarcinoma (112) and are increased in Duke's D stage (13,14). In addition, MMP-2 protein is found at the invasive edge of colon tumors (113). MMP-2 activation has also been shown to be increased in patients with metastases (15). Elevated active MMP-2 was found in the bile of patients with liver metastases when compared to patients with colon cancer but without liver metastases (114). Like MMP-2, increased MMP-9 protein and activity levels are also associated with Duke's staging (13). MMP-9 is more frequently expressed in the invasive regions of advanced tumors (115). Serum MMP-9

levels, although of inactive MMP, are also elevated in patients with liver metastases (114).

As mentioned previously, TIMPs inhibit MMP activation. Although TIMP-1 is capable of affecting the activity of many MMP, TIMP-1 specifically binds to pro-MMP-9, inhibiting its activation [For a review please see: (9)]. Our data show that retinol treatment increases TIMP-1 protein levels in the medium of both cell lines (Fig. 3.6). The increase in media TIMP-1 levels (Fig. 3.6A and B) occurs concomitant with decreased MMP–9 activity in both cell lines (Fig. 3.4). It is possible that TIMP-1 may bind to and prevent the activation of any pro-MMP-9 present in the culture media, thereby inhibiting cell invasion, however no pro-MMP-9 was detected in the tissue culture media (Fig. 3.4). Therefore, the lower MMP-9 activity most likely reflects the decrease in MMP-9 mRNA and protein levels, and not the increase in media TIMP-1 protein, that occurs in response to retinol treatment (Fig. 3.3 and 3.5).

In contrast to what might be expected based on *in vitro* studies (116-118), elevated serum TIMP-1 levels are correlated with a poor disease outcome in clinical studies (119-122). In such cases, TIMP's tumor promoting activities were not linked to its ability to inhibit MMP (123-126). Rather, the poor clinical outcome of patients with elevated TIMP-1 levels is thought to be due to the stimulatory activity of TIMP-1 on cell growth. However, in our study, retinol did not affect cell growth after 24 h of treatment (data not shown), the time at which media levels of TIMP-1 were measured. The link between serum TIMP-1 levels, MMP activity, and clinical outcome requires further study. Because our study used Matrigel-coated Boyden chambers, it lacked the full complement of ECM proteins that occur *in vivo*, therefore future studies will be

conducted utilizing an animal model to assess the effect of retinol on MMP and TIMP-1 levels on colon cancer metastasis.

In conclusion, the present study shows that retinol inhibits colon cell invasion by decreasing MMP activity and increasing TIMP-1 levels *in vitro* through an ATRA and RAR-independent mechanism. While the particular MMP affected varied with cell line, MMP-9 mRNA and protein levels were decreased in both ATRA-resistant cell lines examined. Retinol also increased TIMP-1 levels in conditioned media obtained from both cell lines. The ability of retinol to decrease the metastatic potential of ATRA-resistant colon cancer cell lines suggests that dietary vitamin A supplementation may prevent colon cancer progression.

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Table 3.1. Sequence of primers used for SYBG quantitative real time-PCR

Target Gene	Sense primer (5'-3')	Antisense primer (5'-3')
MMP-1	GATGAAGTCCGGTTTTTCAAAG	GCAGCATCGATATGCTTCAC
MMP-2	CGGAAAAGATTGATGCGGTA	TGCTGGCTGAGTAGATCCAG
MMP-7 (127)	GGATGGTAGCAGTCTAGGGATTAACT	GGAATGTCCCATACCCAAAGAA
MMP-9	ATCCGGCACCTCTATGGTC	CTGAGGGGTGGACAGTGG
MMP-13	CCAGTCTCCGAGGAGAAACA	AAAAACAGCTCCGCATCAAC
TIMP-1 (128)	CTTCTGGCATCCTGTTGTTG	AGAAGGCCGTCTGTGGGT
GAPDH (129)	TGCACCACCAACTGCTAGC	GGCATGGACTGTGGTCATGAG

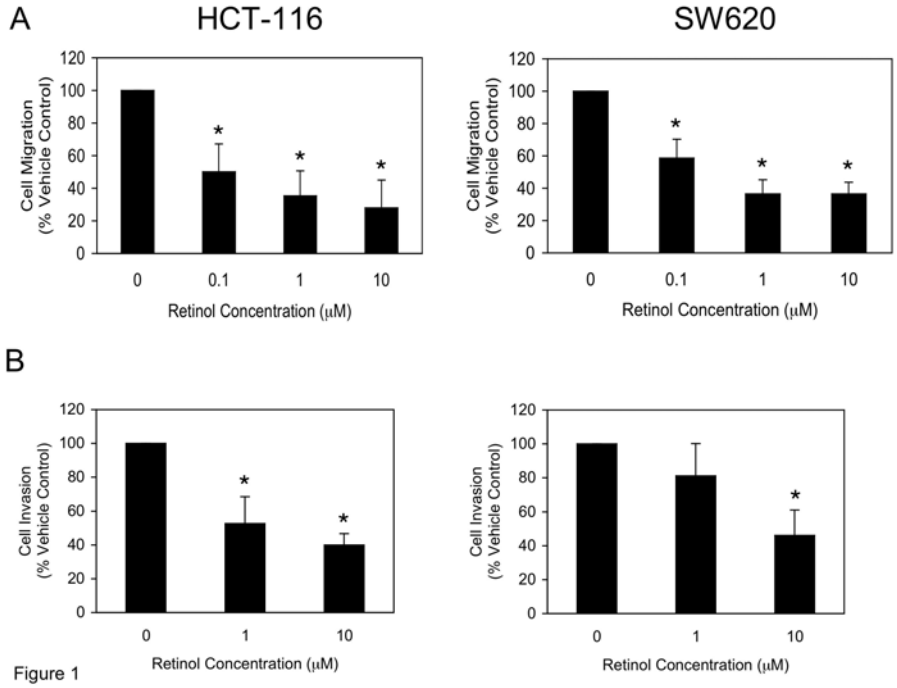

FIGURE 3.1. EFFECT OF RETINOL ON ATRA-RESISTANT COLON CANCER CELL MIGRATION AND INVASION. HCT-116 (left column) and SW620 (right column) cells were serum starved for 48 h before seeding at a density of 1 x 10^5 cells/well. Uncoated Boyden chambers were used to assess the effect of retinol on cell migration (A). The upper portion of the chambers contained 0 (ethanol vehicle control), 0.1, 1, or 10 μM retinol. An 8 μm pore-sized filter separated the cells from a lower chamber containing 10% FBS, which served as a chemoattractant. Cell migration was measured after 8 h by propidium iodide staining. All data are reported as mean ± SEM for three (HCT-116) or five (SW620) experiments. Matrigel-coated Boyden chambers were used to examine the effect of retinol on cell invasion (B). The upper portion of the chambers contained 0 (ethanol vehicle control), 1, or 10 μM retinol and the lower portion contained 20 ng/ml HGF and 10% FBS, which served as chemoattractants. Cell invasion was measured after 24 h by propidium iodide staining as described in Materials and Methods. All data are reported as mean ± SEM for n=3. *Significantly different from control, $P < 0.05$.

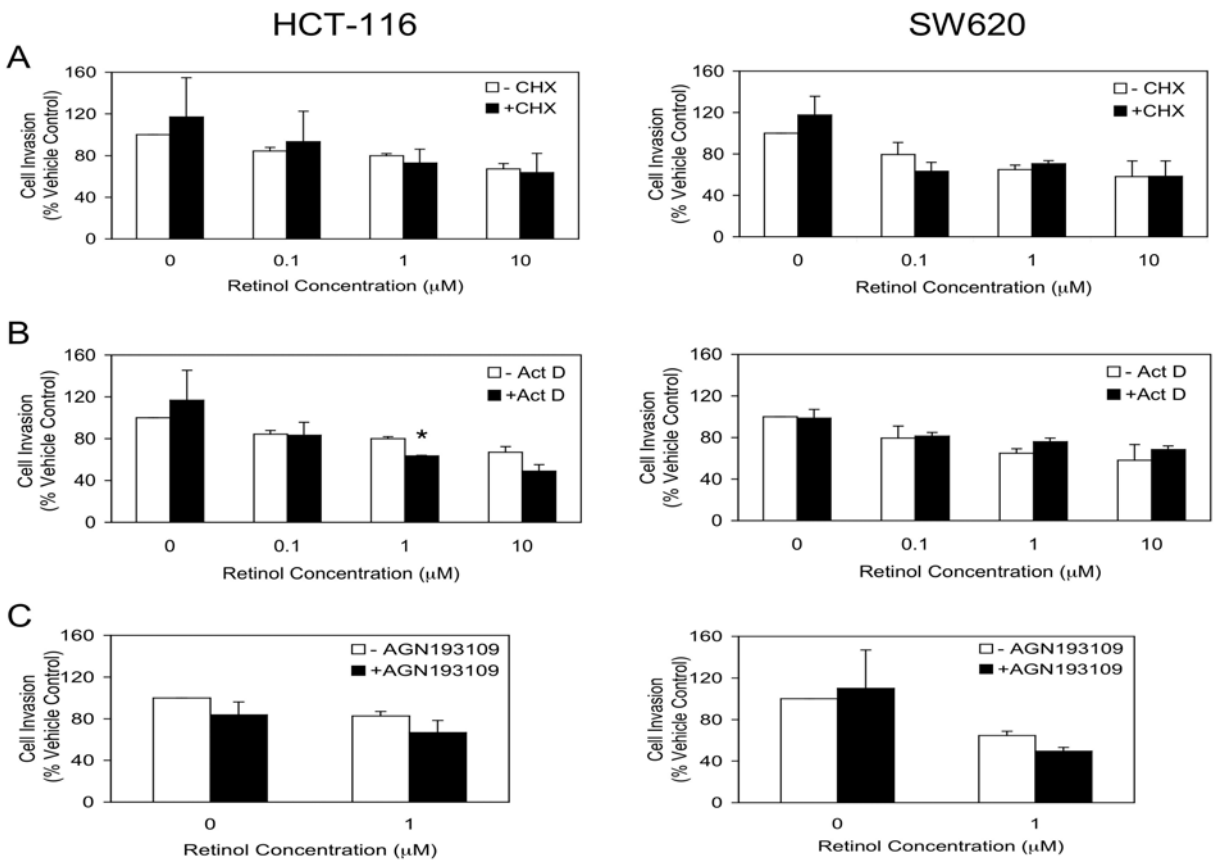

FIGURE 3.2. EFFECT OF CO-TREATMENT WITH RETINOL AND CYCLOHEXIMIDE, ACTINOMYCIN D OR A PAN-RAR ANTAGONIST ON ATRA-RESISTANT COLON CANCER CELL INVASION. HCT-116 (left column) and SW620 (right column) cells were serum starved for 48 h before seeding at a density of 1 x 10^5 cells/well on Matrigel-coated Boyden chambers. The upper portion of the chambers contained 0 (ethanol vehicle control), 0.1, 1, or 10 μM retinol with or without 10 μg/ml cycloheximide (CHX) (A) or 2 μg/ml actinomycin D (Act D) (B). Cells were also treated with 1 μM retinol with and without 10 μM AGN193109 (C). The lower chamber contained 20 ng/ml hepatic growth factor and 10% FBS. Cell invasion was measured after 24 h by propidium iodide staining as described in Materials and Methods. All data are reported as mean ± SEM, n=3. *Significantly different from cells not treated with actinomycin D, $P < 0.05$.

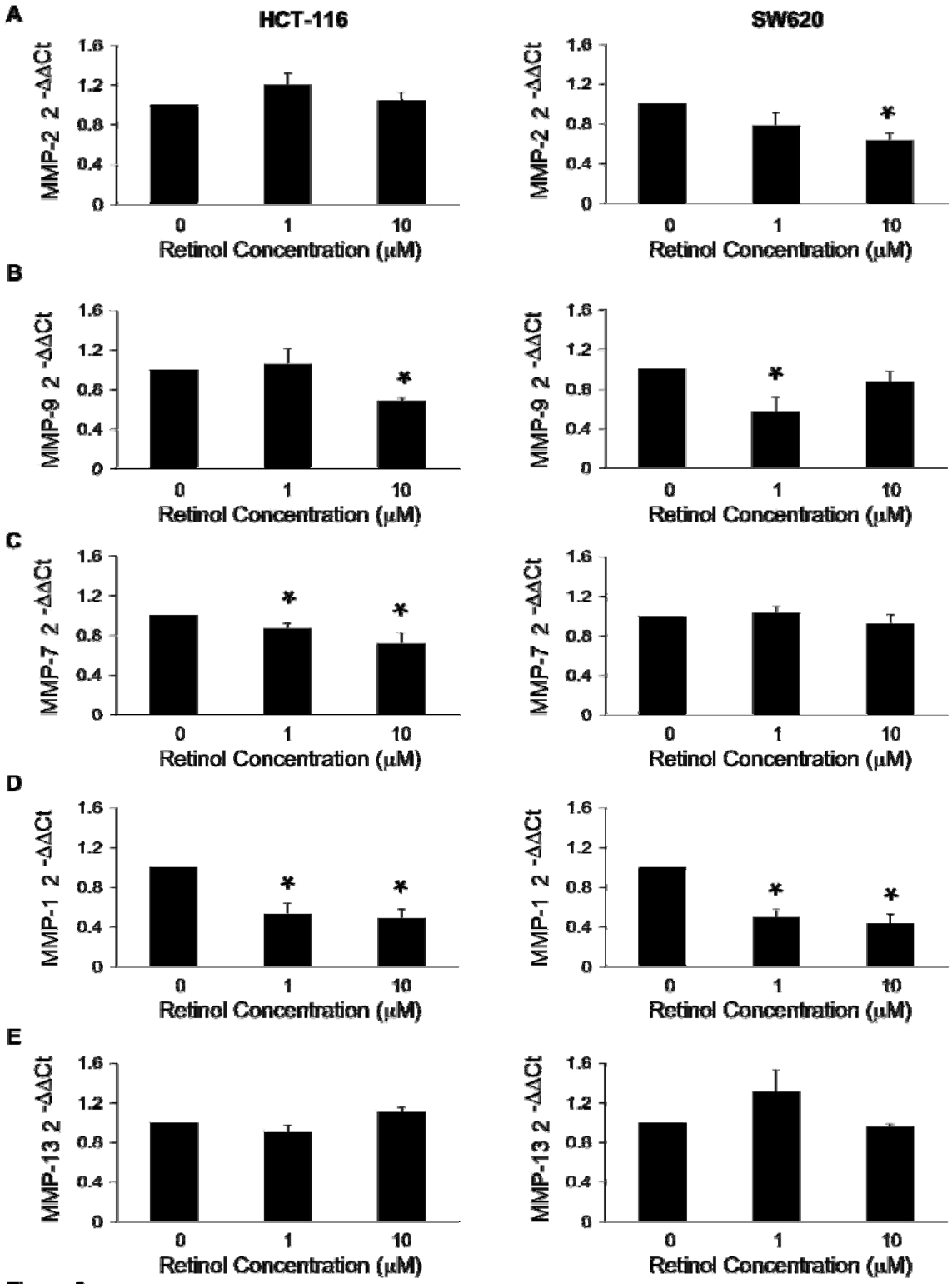

FIGURE 3.3. EFFECT OF RETINOL ON MMP MRNA LEVELS.

FIGURE 3.3. EFFECT OF RETINOL ON MMP MRNA LEVELS. HCT-116 (right column) and SW620 (left column) cells were treated with 0 (ethanol vehicle control), 1, or 10 μM retinol for 24 h. Five different MMP mRNA levels (A: MMP-2, B: MMP-9, C: MMP-7, D: MMP-1 and E: MMP-13) were detected by quantitative real time RT-PCR. Quantitative real time RT-PCR reactions were performed in duplicate. Data are reported as mean ± SEM, n=3. Relative amounts of MMP cDNA were calculated by the comparative CT method. CT values obtained for the different MMP were normalized to corresponding CT values of GAPDH. *Significantly different from control, $P < 0.05$.

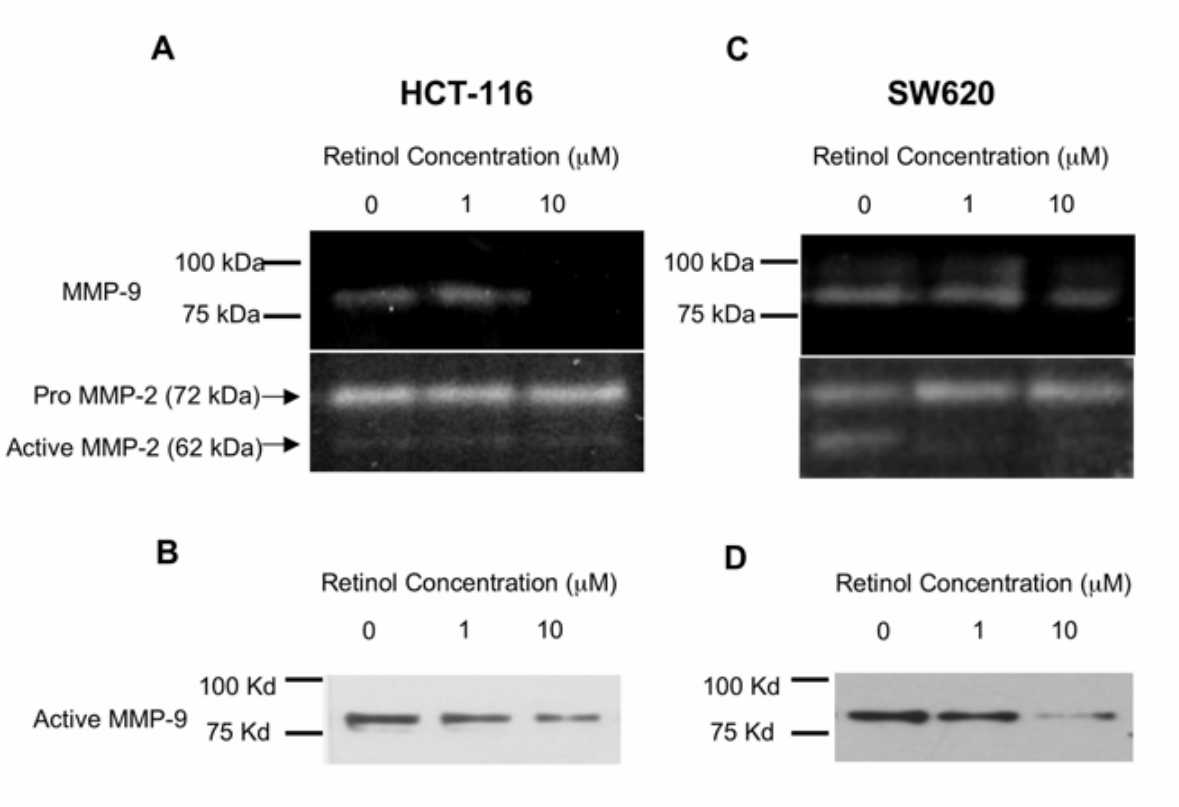

FIGURE 3.4. EFFECT OF RETINOL ON MMP ACTIVITY AND PROTEIN LEVELS. HCT-116 (left columns, A and B) and SW620 (right column, C and D) cells were treated with 0 (ethanol vehicle control), 1, or 10 μM retinol for 24 h. Gelatin zymograms (A and C) displaying the active form of MMP-9 (88-92 kDa) and both the pro and active forms of MMP-2 (72 kDa and 62 kDa, respectively) are shown. Western blots (B and D) displaying active (88-92 kDa) MMP-9 protein levels in serum free conditioned media harvested from cells treated for 24 h with retinol. Pro-MMP-9 (95-98 kDa) was not detected in the conditioned media. Each experiment was repeated three times and one representative experiment is shown.

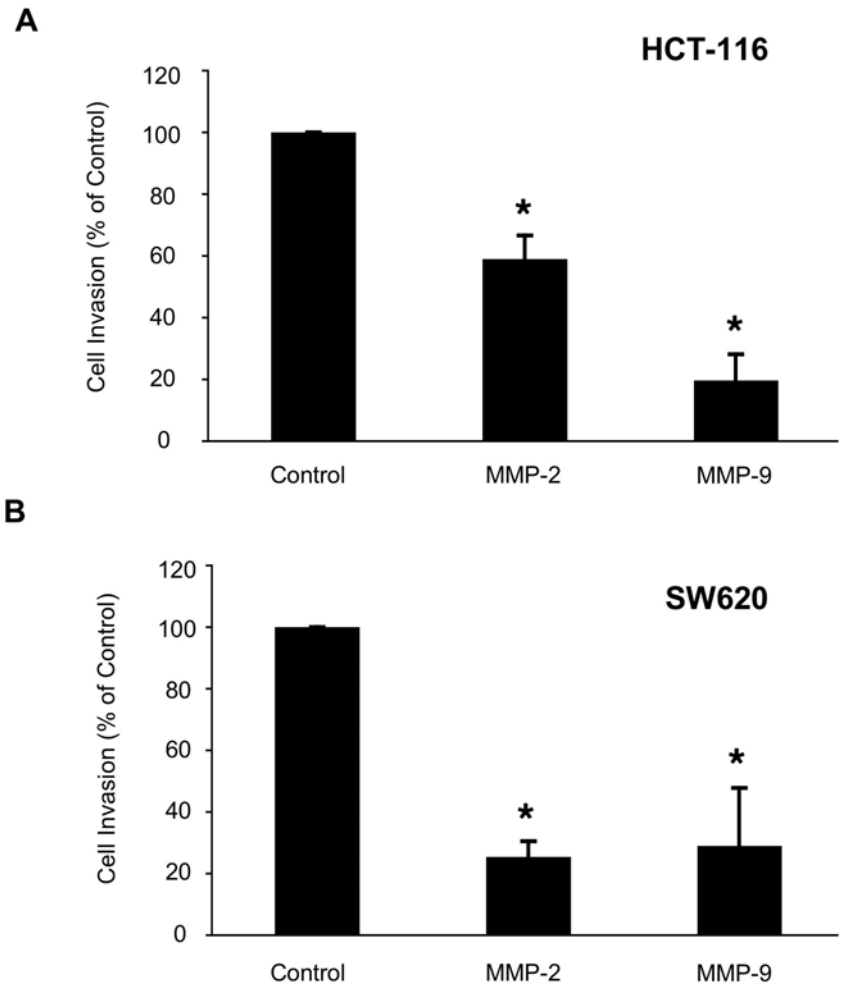

FIGURE 3.5. MMP ANTIBODIES BLOCK THE INVASION OF HCT-116 AND SW620 CELLS. HCT-116 (A) and SW620 (B) cells were serum starved for 48 h before seeding at a density of 1 x 10^5 cells/well on Matrigel-coated Boyden chambers. The upper portion of the chambers contained 10 μg/ml of MMP-2 or -9 antibody in serum free media. The lower chamber contained 20 ng/ml HGF and 10% FBS. Cell invasion was measured after 24 h by propidium iodide staining as described in Materials and Methods. All data are reported as mean ± SEM, n=2. *Significantly different from control, $P < 0.05$.

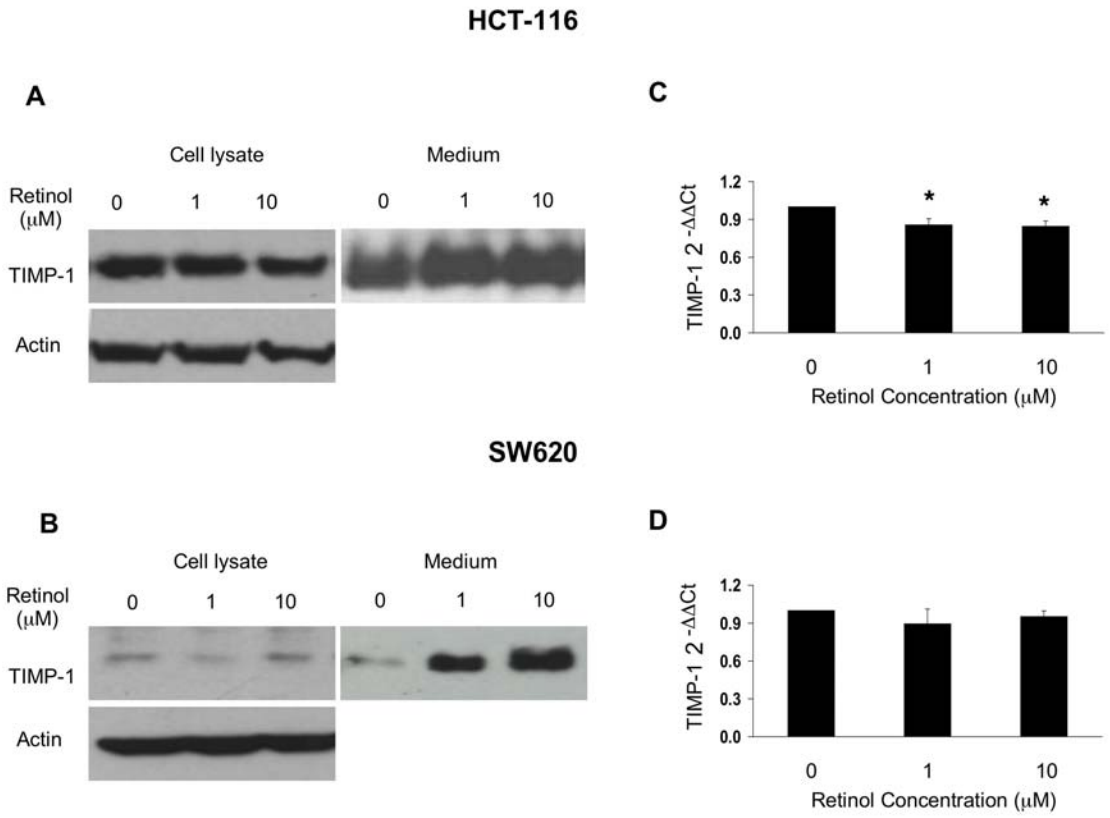

FIGURE 3.6. EFFECT OF RETINOL ON TIMP-1 PROTEIN AND MRNA. HCT-116 and SW620 cells were treated with 0 (ethanol vehicle control), 1, or 10 μM retinol for 24 h. Proteins were collected from HCT-116 (A) and SW620 (B) cells and culture media as described in Materials and Methods. TIMP-1 protein was detected in cell lysates and conditioned media. β-Actin is shown as a loading control for the cell lysates. TIMP-1 mRNA levels in HCT-116 (C) and SW620 (D) cells were detected by quantitative real time RT-PCR. Quantitative real time RT-PCR reactions were performed in duplicate. Data are reported as mean ± SEM, n=3. Relative amounts of TIMP-1 cDNA were calculated using the comparative CT method. CT values obtained for TIMP-1 were normalized to corresponding CT values of GAPDH. *Significantly different from control, $P < 0.05$.

Chapter 4: Retinol Decreases Phosphatidylinositol 3-Kinase Activity in Colon Cancer Cells

ABSTRACT

Previously, we showed that retinol inhibited *all-trans*-retinoic acid (ATRA)-resistant human colon cancer cell invasion via a retinoic acid receptor-independent mechanism. Because phosphatidylinositol 3-kinase (PI3K) regulates cell invasion, the objective of the current study was to determine if retinol affected PI3K activity. Following 24 h of serum starvation, the *all-trans*-retinoic acid resistant human colon cancer cell lines HCT-116 and SW620 were treated with 0, 1, or 10 μM retinol. Thirty min of retinol treatment resulted in a significant decrease in PI3K activity in both cell lines. To determine the mechanism by which retinol reduces PI3K activity, the levels and heterodimerization of the regulatory subunit, p85, and the catalytic subunit, p110, of PI3K were examined. Retinol treatment did not alter p85 or p110 protein levels or the heterodimerization of these subunits at any time point examined. To determine if retinol affected the ability of PI3K to phosphorylate the substrate, phosphatidylinositol (PI), PI3K was immunoprecipitated from control cells and incubated with 10 μg PI and increasing concentrations of retinol or 10 μg retinol and increasing concentrations of PI. Retinol decreased PI3K activity in a dose-responsive manner and increased PI suppressed the inhibitory effect of retinol on PI3K activity. Finally, the PI3K inhibitor, LY294002, mimicked the ability of retinol to decrease cell invasion. Computational modeling revealed that retinol may inhibit PI3K activity in a manner similar to that of wortmannin.

Thus, a decrease in PI3K activity due to retinol treatment may confer the ability of retinol to inhibit ATRA-resistant colon cancer cell invasion.

INTRODUCTION

Colorectal cancer is the third most common cancer and cause of death due to cancer in the United States. The five-year survival rate for colorectal cancer patients with metastasis is only 10% (130). Death due to colorectal cancer is generally caused by hepatic metastasis of the primary tumor, rather than the primary tumor itself (7). The retinoids, a group of compounds consisting of vitamin A (retinol), its natural metabolites, and several synthetic compounds, have been shown to inhibit metastasis in a variety of model systems. For example, dietary retinyl palmitate decreased malignant melanoma metastasis in mice (131). *All-trans*-retinoic acid (ATRA) decreased breast cancer (132), gastric cancer (133), and colon cancer invasion (134) *in vitro* and rhabdomyosarcoma metastasis in rats (135). Also, retinol decreased hepatic metastasis in a hamster model of pancreatic ductal carcinoma (136).

Dietary retinyl-esters are converted to retinol in the intestinal lumen. Thus, dietary vitamin A supplementation can elevate retinol levels in the colon. Once absorbed, retinol is re-esterified and transported to the liver, the major site of vitamin A storage. Although serum retinol levels in non-vitamin A deficient animals vary from 1-2 μM, regardless of supplementation status, [for a review please see: (43)], hepatic retinol levels increase in response to supplementation and values in excess of 90 μM have been reported (44).

Phosphatidylinositol 3-kinases (PI3Ks) catalyze the phosphorylation of the 3-OH position of the inositol head groups of the phosphatidylinositol (PI) lipids, phosphatidylinos--itol(4)phosphate [PI(4)P], and phosphatidylinositol(4,5)bisphosphate [PI(4,5)P2] to generate PI(3)P, PI(3,4)P2 and PI(3,4,5)P3, respectively (16). PI3Ks can be divided by three different main classes based on their lipid substrate and structure. Class IA PI3K consists of the regulatory subunit, p85, and the catalytic subunit, p110, and catalyzes the phosphorylation of all three substrates, PI, PI(4)P, and PI(4,5)P2.

Activation of PI3K is associated with increased cell invasion and tumor metastasis (137,138). In addition, PI3K activity is increased in human colon cancer (139). PI3K activity is regulated by altering the protein levels or heterodimerization of the p85 regulatory and p110 catalytic subunits of PI3K. ATRA has been shown to affect PI3K activity by increasing p110β mRNA (140) and protein levels or p85α mRNA and phosphorylation (141). In addition, cellular retinol binding protein I (CRBPI) inhibits PI3K/Akt signaling by decreasing the heterodimerization of p85 and p110 subunits of PI3K (142).

ATRA inhibited airway smooth muscle cell migration by inhibiting PI3K/Akt-dependent actin cytoskeleton reorganization (143). Previously, we showed that retinol decreased the invasion of ATRA-resistant human colon cancer cells via a novel RAR-independent mechanism (144). The objectives of present study were to determine if retinol affected PI3K activity in ATRA-resistant human colon cancer cells and if inhibition of PI3K decreased the invasion of these cells.

MATERIALS AND METHODS

Tissue Culture

The human colorectal carcinoma cell line, HCT-116, and the human colorectal adenoma cell line, SW620, were obtained from the American Type Culture Collection (Manassas, VA) and grown in DMEM in a humidified atmosphere at 37°C with 5% CO_2. Media were supplemented with 10% FBS (fetal bovine serum) and antibiotics (1000 U/mL penicillin and 1000 μg/mL streptomycin). All cells were serum starved for 24 h before retinol treatment. Retinol was added to DMEM medium containing 10% FBS and antibiotics. Retinol was dissolved in ethanol and all retinoid manipulations were performed under subdued light.

PI3K Activity Assay

HCT-116 and SW620 cells were plated at a density of 1 x 10^6 cells/100 mm dish and 2 x 10^6 cells/100 mm dish, respectively. Cells were treated with 0, 1, and 10 μM retinol for 30 min following 24 h of serum starvation. Cells were lysed with cell lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 1 μg/ml Leupeptin and 1 mM PMSF). Cell protein lysate concentrations were determined using the BioRad DC protein assay kit (Hercules, CA). Total PI3K protein was detected using p85 antibody as described in the Western Blot Analysis section, below. Total PI3K protein was normalized to β-actin. PI3K proteins were

immunoprecipitated by incubation of 400 μg of whole cell protein lysate with 4 μg of IRS-1 antibody (Upstate, #06-248, Lake Placid, NY) overnight at 4°C. The next day, 50 μl of protein A/G plus-agarose beads (Santa Cruz Biotechnology, #sc-2003, Santa Cruz, CA) were added to the lysate and incubated for 2 h at 4°C.

PI3K activity was determined as described previously (145) with minor modification as follows. The beads were washed twice with 1% NP-40 and once with each of the following: PBS, LT (lithium Tris) buffer [0.5 M LiCl and 200 mM Tris-HCl (pH 7.5)], TEN (Tris, EDTA, sodium chloride) buffer (20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 100 mM NaCl), and TGN (Tris, EGTA, sodium chloride) buffer (20 mM Tris-HCl (pH 7.5), 1 mM EGTA, and 100 mM NaCl). The washed beads were resuspended in 40 μl TGN buffer. Immunoprecipitated PI3K was incubated with 10 μg phosphatidylinositol PI (Avanti Polar Lipid, Alabaster, AL) for 10 min at room temperature followed by incubation with 10 μCi of [γ-^{32}P] ATP and 20 mM $MgCl_2$ for 20 min at room temperature. The reaction was stopped with 100 μl 1N HCl. The samples were centrifuged for 1 min at 15,000 x *g*. Following centrifugation, the supernatant was extracted with a 1:1 solution of chloroform:methanol and the lower fraction spotted on TLC (thin layer chromatography) plates (Fisher Scientific Company, Houston TX). Before use, the TLC plate was treated with coating solution (40% methanol, 1.2 mM EDTA and 1% potassium oxalate) for 30 min and dried overnight. TLC separation buffer consisted of chloroform:methanol:30% ammonium hydroxide:H_20 = 129:114:15:21. Dried TLC plates were exposed to film at –70°C for 3 to 5 d.

Western Blot Analysis

Cells were plated at a density of 1 x 10^6 cells/100 mm dish. Cells were treated with 0, 1, and 10 μM retinol for 30, 60, 120 and 240 min after 24 h of serum starvation. Western blot analysis was performed as described previously (146). Protein (50 μg) was electrophoresed through 8% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were incubated with a 1:100 dilution of p110 antibody (Santa Cruz Biotechnology, #sc-8010, Santa Cruz, CA) in 5% BSA in TBST (Tris-buffered saline, Tween) overnight at 4°C or with a 1:100 dilution of p85 antibody (Santa Cruz Biotechnology, #sc-423, Santa Cruz, CA) in TBST overnight at 4°C.

Immunoprecipitation of PI3K

Cells were plated as described above in the PI3K Activity Assay section. Cells were treated with 0 and 10 μM retinol for 5, 10, 20, 30, 60, and 120 min after 24 h of serum starvation. Cells were lysed with cell lysis buffer as described in the PI3K Activity Assay section, above. PI3K protein was immunoprecipitated by incubation of 200 μg of whole cell protein lysate with 2 μg of p110 antibody overnight at 4°C. The following day, 25 μl of sepharose 4A beads (50 mg/ml) (Amersham Biosciences, Piscataway, NJ) were added to the lysate prior to incubation for 2 h at 4°C. The beads were washed twice with 1% NP-40 and once with PBS. Protein samples were subjected to western blot analysis as described above.

PI3K Substrate Competition Assay

Cells were plated as described above in the PI3K Activity Assay section. Cells were treated with 10% FBS for 30 min following 24 h of serum starvation. Cells were not treated with retinol prior to lysis. Cells were lysed with cell lysis buffer as described in the PI3K Activity Assay section, above. PI3K proteins were immunoprecipitated by incubation of 400 μg of whole cell protein lysate with 2 μg of p110 antibody overnight at 4°C. Following incubation, 25 μl of sepharose 4A beads (50 mg/ml) were added for 2 h at 4°C. The beads were washed twice with 1% NP-40 and once with each of the following; PBS, LT, TEN, and TGN. The washed beads were resuspended in 50 μl TGN buffer. Ten μl samples were subjected to western blot analysis for p110 levels to serve as an internal loading control, as described above. Immunoprecipitated PI3K was incubated with 10 μg PI, retinol vehicle (ethanol) or increasing concentrations of retinol (0, 10, 20, 50, and 100 μg; corresponding to 0, 0.6, 1.2, 2.9, and 5.8 μM retinol, respectively) for 10 min at room temperature prior to the addition of 10 μCi of [γ-^{32}P] ATP and 20 mM $MgCl_2$ for 20 min at room temperature. In addition, immunoprecipitated PI3K was incubated with 10 μg retinol and increasing concentrations of PI (10, 20, and 50 μg). The total volume of ethanol vehicle was the same in all samples, regardless of retinol concentration. Lipid extraction and TLC were performed as described above in the PI3K Activity Assay section.

Invasion Assays

Cells were serum starved for 24 h before seeding at a density of 1 x 10^5 cells/well onto Boyden chambers coated with Matrigel. The upper portion of the chambers contained 0 (ethanol vehicle control) or 10 μM retinol and 0, 10 or 50 μM of the PI3K inhibitor, LY294002. DMEM containing 10% FBS was added to the lower portion of each chamber to serve as a chemoattractant. After 24 h, all cells were removed from the upper chamber using a cotton swab. HCT-116 cells that had migrated through the membrane were stained with crystal violet. SW620 cells that had migrated through the membrane were fixed in methanol prior to staining with propidium iodide. The bottom of the membrane was examined microscopically and the number of stained cells present in ten random fields of view counted to determine cell invasion as described (96).

Computational Methods

The Hyperchem 7.5 modeling environment was used to perform a series of calculations on retinol, wortmannin and a fragment of PI. To decrease the computational complexity the native PI structure was fragmented by decreasing both of the ester chains to a single methyl group. This model compound should remain representative of the larger structure as the activity of PI at PI3K is focused near the cyclohexyl and phosphate groups and not at the non-polar side chains, which instead interact with the cell membrane (147). Structure pre-optimization was conducted using semi-empirical quantum mechanics with a MNDO/d parameterization. Resulting structures were then carried forward to a more complex *ab initio* optimization using a

RHF/6-31G(d) model chemistry to generate not only a starting geometry for further calculations but more importantly a set of atomic charges for each of the atoms.

Molecular dynamics was employed to interrogate the conformational space of the ligands via a series of simulated annealing experiments. Since the compounds were all relatively small organics, all dynamics calculations were performed using an MM+ force field with electrostatic contributions to the potential energy term calculated using the atomic charges generated from the *ab initio* optimization. The annealing cycle was conducted using a time step of 0.0005 ps and composed of a 5 ps heating step where the structure was taken from 0°K to 800°K, followed by a 30 ps production phase at constant temperature, before a final 10 ps cooling phase from 800°K back to 0°K. This final cooled structure was then energy optimized and saved before becoming the starting structure for a subsequent annealing cycle. The procedure was repeated 100 times to generate 100 candidate conformations for retinol, wortmannin and the PI fragment. The 100 conformations were sorted based on distances, angles and torsion angles of various moieties to produce a set of conformational families for each compound. An electrostatic potential isosurface was generated for the lowest energy structure of each of these conformation families. The isosurface provided a means of assessing the electrostatic character of each compound and facilitates comparisons of their potential substrate interactions by highlighting locations of high reactivity. The ligands in this study were not docked to the p110 protein, but instead compared to each other to examine similarities that might help elucidate their potential interaction with PI3K.

Statistical Analysis

Quantitation was performed with Biorad Quantity One software. Values shown are the mean ± SEM of three independent experiments. All data are expressed relative to vehicle control. All statistical tests were performed using SPSS (Apache Software Foundation, Wilmington, DE, version 12.0 for Windows). Data were analyzed using two-tailed t-tests comparing each retinol concentration to control (0 μM). Results were considered significantly different at $P < 0.05$.

RESULTS

Retinol decreases PI3K activity.

Previously, we showed that retinol inhibited the invasion of HCT-116 and SW620 ATRA-resistant human colon cancer cell lines (144). Because PI3K plays a regulatory role in tumor metastasis (137,138), we examined the effect of retinol on PI3K activity in intact cells, treated with 0, 1 and 10 μM retinol for 30 min immediately prior to harvest. Retinol decreased PI3K activity in both the HCT-116 and SW620 cell lines (Fig. 4.1A and B). Specifically, in the HCT-116 cell line, 1 and 10 μM retinol significantly decreased the activity of PI3K to 59.8 ± 11.4 and 70.3 ± 7.7% of vehicle control, respectively (Fig. 4.1A). Treatment with 1 μM retinol trended to reduce PI3K activity in the SW620 cell line to 70.8 ± 11.4% of vehicle control (Fig. 4.1B). Treatment with 10 μM retinol also significantly reduced PI3K activity in the SW620 cell line to 71.6 ± 5.9% of vehicle control (Fig. 4.1B). There were no differences in PI3K activity between

the 1 and 10 μM retinol treatments in either cell line, indicating that maximal PI3K inhibition may have been achieved following treatment with 1 μM retinol. These data indicate that treatment of intact ATRA-resistant colon cancer cells with retinol rapidly decreases PI3K activity.

Retinol does not decrease p110 and p85 protein levels or heterodimerization.

Western blot analysis and immunoprecipitation were used to determine if retinol decreased PI3K activity by reducing PI3K protein levels or if retinol affected the heterodimerization of the p85 and p110 subunits of PI3K. As shown Fig. 1, PI3K activity was decreased after as little as 30 min of retinol treatment. In addition, we showed previously that the ability of retinol to inhibit cell invasion was independent of transcription and translation (144). Cells were treated with retinol from 30 to 240 min to determine the effect of retinol on p110 and p85 protein levels. Retinol treatment did not alter either p110 or p85 protein levels at any time point examined (Fig. 4.2 A, B, D and E).

Heterodimerization of p85 and p110 activates PI3K (16). To determine retinol's effect on the interaction of p85 and p110, cells were treated with retinol for 5 to 120 min. p110 antibody was used for immunoprecipitation and p85 antibody was selected for immunoblotting to detect the binding of p110 to p85. Retinol treatment did not change p85 and p110 heterodimerization at any time point examined (Fig. 4.2 C and F). These data indicate that retinol does not exert its inhibitory effect on PI3K activity by decreasing p85 or p110 protein levels or their heterodimerization.

Retinol decreases PI3K activity by inhibiting PI/PI3K interaction.

To elucidate whether retinol decreased PI3K activity by inhibiting the ability of the PI3K substrate, PI, to interact with PI3K, we immunoprecipitated PI3K from non-retinol-treated cell lysates and measured the activity of this purified PI3K in the presence of increasing amounts of retinol. As can be seen in Fig. 4.3, the activity of PI3K was decreased by retinol in dose-dependent manner. The activity of PI3K in HCT-116 and SW620 cells was significantly decreased to 21.3 ± 10.9 and 41.8 ± 19.7% of vehicle control, when incubated with 10 μg retinol (0.6 μM retinol) and 10 μg PI, respectively. In addition, the activity of PI3K was further reduced to 10.3 ± 4.3, 6 ± 3.6, and 6.4 ± 3.4% of vehicle control by incubation with 20, 50, and 100 μg retinol, respectively (corresponding to 1.2, 2.9, and 5.8 μM retinol), in the HCT-116 cell line. Similarly, in SW620 cells, the activity of PI3K was decreased to 14.6 ± 6.6, 11.6 ± 8.8, and 11.5 ± 9.1% of vehicle control by incubation with 20, 50, and 100 μg retinol, respectively (corresponding to 1.2, 2.9, and 5.8 μM retinol). Retinol inhibits PI3K activity to a greater extent when PI3K is purified (Fig. 4.3) than when whole cells are treated with retinol (Fig. 4.1). Thus, it is conceivable that the metabolism of retinol or the sequestration of retinol by binding proteins, such as CRBP-I, may modulate the ability of retinol to inhibit PI3K activity in intact cells. Importantly, the ability of retinol to more significantly inhibit the activity of immunoprecipitated PI3K demonstrates that retinol is directly affecting the enzyme-substrate interaction.

To determine if the ability of retinol to decrease PI3K activity was suppressed by PI, immunoprecipitated PI3K from non-retinol-treated HCT-116 cell lysates was incubated with 10 μg retinol and increasing concentrations of PI. As can be seen in Fig. 4.3C, the decrease in PI3K activity in response to retinol was suppressed by incubation with 50 μg of PI. Specifically, 10 μg retinol (0.6 μM retinol) decreased PI3K activity to 38.9 ± 15.9 % of vehicle control. However, when treated with 10 μg retinol and 50 μg PI, PI3K activity increased to 110.0 ± 20.8 % of vehicle control. These data suggest that retinol inhibits the interaction of PI3K with its substrate PI.

Retinol and a PI3K inhibitor both decrease cell invasion.

The PI3K inhibitor, LY294002, was used to determine if PI3K played a role in the invasion of the HCT-116 and SW620 cell lines. As can be seen in Fig. 4.4, HCT-116 and SW620 cell invasion was decreased following treatment with retinol and LY294002. Cell invasion was significantly decreased to 36 ± 6.2% by 10 μM retinol and to 37 ± 13.3 and 28 ± 10.3% by 10 and 50 μM LY294002, respectively, in the HCT-116 cell line (Fig. 4.4A). In the SW620 cell line, invasion was significantly decreased to 31.6 ± 5.8% by 10 μM retinol and to 20.5 ± 3.4 and 9.7 ± 0.8% by 10 and 50 μM LY294002, respectively (Fig. 4.4B). Previously, we showed that cell number was not affected after 24 h of treatment with retinol (144). These data indicate that PI3K is involved in the invasion of these ATRA-resistant colon cancer cell lines. These results, together with those in Fig. 4.3, suggest that retinol may decrease cell invasion by inhibiting the activity of PI3K.

Retinol and wortmannin exhibit similar electrostatic potential surfaces.

Extensive molecular modeling in the form of both *ab initio* calculations and molecular dynamics stimulations were performed to provide a better understanding of the reactivity of retinol, PI and wortmannin. As expected the *ab initio* calculations revealed a number of non-zero atomic charges that were indicative of various degrees of polarity within the ligands. The most polar of the three ligands was the PI fragment as there were a number of acidic hydrogens on the alcohol groups of the cyclohexyl ring as well as a significant charge gradient near the phosphate moiety. Wortmannin also contained a number of electron dense areas, but fewer acidic hydrogens overall as the most reactive hydrogen was the one attached directly to the five-membered furan ring. These results parallel those reported by x-ray crystallography that suggested this particular hydrogen atom was directly involved with PI3K complexation (148). Retinol was observed to contain only one acidic hydrogen atom, the one located on the alcohol group, and thus based on polarity alone might be considered the least reactive ligand as a majority of the molecule is encompassed by a relatively non-polar hydrocarbon ring and hydrocarbon chain.

The simulated annealing procedure generated conformational families for retinol that could be categorized by the distance between the terminal alcohol group and the ring, the angle the ring formed with the midpoint of the carbon chain and the hydroxyl group, and the torsion angles along the unsaturated hydrocarbon chain. The vast majority of the structures formed one major family that showed folding of the hydrocarbon chain inward toward the ring as the pi orbitals of the unsaturated carbon-carbon double bonds aligned to form a ring-like structure. The elongated conformation that left the hydroxyl

group completely extended away from the ring was also identified, albeit with less frequency. Although it lacked orbital stabilization, this elongated conformation was relatively low in energy because of the low steric interactions caused by the extension of the hydrocarbon chain.

The PI fragment results were categorized by the torsion angles near the phosphate moiety and the relative spatial distance between the two ester linkages and the hydroxyl laden ring. While the interaction of the ester functionalities with the ring is favorable in the model compound, structures that adopted this form were eliminated from consideration because the active form of PI has the two lengthy carbon chains extended away from the binding site, and located in the cell membrane (147), not folded over the ring. The presence of these folded conformations was simply an artifact of the adoption of the model fragment. Instead, only the PI structures that maintained their extended nature were considered for the subsequent calculations. Not surprisingly only one major conformational family was identified for wortmannin as a majority of the compound's structure is built around a relatively rigid ring scaffold.

Electrostatic potential surfaces for the most prominent conformations of the compounds are presented in Fig. 4.5. Here, areas of positive potential are depicted by the green wire frame whereas negative potentials are shown in purple. For reference the potential is displayed over the tube structure of the compound with carbon atoms in blue, oxygen atoms in red, hydrogen atoms in white and phosphorus shown in yellow. The first surface in Fig. 4.5A shows the reactive hydrogen atom of wortmannin (white arrow) located between two relatively negative potential areas created by the lone pairs of

electrons on the oxygen atoms of the furan and neighboring ring (red arrows). The distance across the positively charged pocket is calculated to be approximately 3.5Å.

A typical surface for the PI fragment is shown in Fig. 4.5C. In this case, the numerous reactive hydroxyl atoms lie nestled between a number of negative zones created by the various hydroxyl oxygen atoms and a large negative charge created by the doubly bonded oxygen atom of the phosphate. Measuring across the gap from the potential minimum located near the phosphate to the nearest neighboring negative zone places a relatively acidic hydrogen atom in a pocket of about 5.4 Å. Thus, wortmannin and PI appear to exhibit some similarity in their electrostatic surface in that there are alternating positive and negative zones that surround relatively reactive hydrogen atoms. However, the electron distribution is very different between the two as wortmannin has only one reactive hydrogen atom that is inserted between two large electron dense areas creating only one pocket of high reactivity whereas the PI fragment has that potential spread much more evenly around the ring.

Comparison of retinol's electrostatic potential surface (Fig. 4.5B) to the other two suggests that retinol has a more similar potential profile to wortmannin than to PI. The folded ring places the lone reactive hydrogen atom (white arrow) between two electron dense areas created by the hydroxyl oxygen atom (red arrow) and the pi orbitals of the unsaturated carbon chain (blue arrow). The distance across this pocket is also quite similar to that calculated for wortmannin at approximately 3.6 Å. It should be noted that the opposing purple area in Fig. 4.5B is merely due to the symmetry of the pi orbitals, not from a separate atom or grouping of atoms. Previous work has shown that wortmannin interacts with ATP binding site of the p110 catalytic subunit of PI3K (149).

Therefore, based on this computational data it is feasible that retinol interacts with PI3K in a manner reminiscent to that of wortmannin.

DISCUSSION

Previous work in our laboratory has shown that retinol inhibited ATRA-resistant colon cancer cell invasion (144). The current study demonstrates that retinol treatment decreases PI3K activity in both HCT-116 and SW620 ATRA-resistant human colon cancer cell lines (Fig. 4.1). As little as 30 min of retinol treatment results in a significant decrease in PI3K activity in both cell lines. The ability of retinol to inhibit PI3K activity is not due to reduced p85 regulatory subunit or p110 catalytic subunit levels or to a decrease in the heterodimerization of these two proteins (Fig. 4.2). Rather, retinol treatment decreases PI3K activity by inhibiting PI3K and PI interaction (Fig. 4.3) in purified preparations of PI3K obtained by immunoprecipitation of this enzyme from non-retinol-treated cells. Furthermore, both retinol and LY294002 decrease cell invasion (Fig. 4.4). Finally, retinol and the PI3K inhibitor, wortmannin, exhibit similar electrostatic potential surfaces (Fig. 4.5). Taken together, these data suggest that retinol decreases the invasion of colon cancer cells by inhibiting PI3K, potentially in a manner similar to that of wortmannin.

To our knowledge, this study is the first to show that retinol inhibits PI3K activity. Several studies have shown that ATRA, a metabolite of retinol, alters PI3K activity. However, the direction of the affect depends on cell type. For example, ATRA increased PI3K activity in SH-SY5Y human neuroblastoma (141,150), NIH3T3

mouse fibroblast (151), cultured vascular endothelial (140), HL-60 human promyelocytic leukemia (152-154) and human myeloid leukemia cells (155). In contrast, ATRA decreased PI3K activity in vascular smooth muscle cells (143) and decreases Akt activity, a target of PI3K, in F9 murine teratocarcinoma cells (156), the breast cancer cell lines MCF-7, SKBR3 and ZR-75 (157), and in the head and neck cancer cell line, SqCC/Y1 (158).

The effect of ATRA on PI3K activity has been shown to be mediated by RAR. For example, the effect of ATRA on Akt activity was not observed in RARγ-null F9 cells (158). Interestingly, RARα has been shown to bind with p85 in vascular smooth muscle cells (143). However, the ability of retinol to inhibit PI3K is not RAR-dependent because the cell lines used in the current study lack some or all RAR (31,159-161) and retinol is not a ligand for RAR. In addition, the SW620 cell line did not convert retinol to ATRA and the HCT-116 cell line synthesized only very small amounts of ATRA from retinol (162). The small amount of ATRA made by the HCT-116 cell line did not activate RAR (162). In support the RAR-independent effects of retinol on PI3K activity, previous work from our laboratory showed that the ability of retinol to inhibit cell invasion (144) and cell growth (162) was RAR-independent. In addition, here we demonstrate that retinol decreases the activity of PI3K purified from non-retinol treated cells by immunoprecipitation indicating that retinol itself, not a metabolite, inhibits PI3K activity.

Transfection of MTSV1-7 breast epithelial cells with CRBP-I inhibited PI3K activity by decreasing p85 phosphorylation resulting in decreased p85 and p110 heterodimerization (142). Phosphorylation of p85 promoted p85 and p110 association

and inhibiting p85-p110 association decreased PI3K/Akt signaling (142). In the present study, we measure PI3K activity directly and show that retinol does not decrease p110 or p85 levels or the interaction between these two proteins to reduce PI3K activity (Fig. 4.2). p85 phosphorylation levels were not examined because a decrease in p85 phosphorylation would be reflected in lower p85 and p110 heterodimerization. No retinol was added to the cell culture medium in the study by Farias *et al.* (142) other than that present in the calf serum supplement. We cannot rule out the possibility that CRBP-I is involved in the ability of retinol to inhibit PI3K. However, CRBP-I inhibits p85 phosphorylation resulting in decreased p85/p110 binding (142) and p85/p110 binding was not affected by retinol in the present study. Also, because retinol inhibits the activity of purified PI3K, we believe that while CRBP-I is present in HCT-116 cell line (163), CRBP-I might merely supply retinol, facilitating the inhibition of PI3K, rather than playing a regulatory role.

The PI3K gene is often overexpressed or mutated contributing to tumor progression in breast, colon, and several other cancers [For a review please see: (22)]. In addition, PI3K regulates multiple signaling components for tumorigenesis including cytoskeletal organization, metastasis, invasion, and angiogenesis [For a review please see: (164)]. The ability of LY294002 to decrease the invasion of ATRA-resistant colon cancer cell lines indicates that the PI3K signaling pathway is involved in the invasion of these cells (Fig. 4.4). Other studies have also shown (149) that inhibition of PI3K activity suppressed the invasion and metastasis of cancer cells. For example, Bikunin decreased human ovarian cancer cell metastasis and invasion through suppression of PI3K (165). Down regulation of the PI3K/Cdc42/Rac 1 signaling pathway by Emodin

also correlated with suppression of tumor cell migration (166). In addition, Topotecan inhibited vascular endothelial cell migration through inhibition of the PI3K/Akt signaling pathway (167).

PI3K is an attractive target for drug development to increase apoptosis, decrease proliferation and block tumor progression [For a review please see:(168,169)]. *Ab inito* and molecular dynamics calculations revealed that retinol and wortmannin exhibit similar electrostatic potential surfaces (Fig. 4.5). Importantly, wortmannin also suppresses PI binding to p110 (170), in a manner similar to that observed for retinol (Fig. 4.3). However, it should be noted that this computational modeling does not strictly discount the possibility that retinol interacts directly in the substrate binding domain of p110 in a manner similar to that of PI. Further computational investigation culminating in a large scale docking study would be required to provide more conclusive proof. Instead, these results simply support the possibility that an alternate binding method for retinol to PI3K may exist and that there are significant electrostatic similarities between retinol and the known PI3K inhibitor, wortmannin.

In conclusion, the present study shows that retinol decreases PI3K activity by decreasing the interaction between PI3K and PI. Activation of the PI3K/Akt pathway contributes to tumor metastasis and resistance to chemotherapy [for a review please see, (171)]. Treatment of ATRA-resistant colon cancer cell lines with retinol or a PI3K inhibitor resulted in decreased cell invasion indicating that retinol may reduce cell invasion by inhibiting PI3K activity. Because elevated retinol levels can be achieved in the colonic lumen and liver via dietary vitamin A supplementation, retinol, or a synthetic

derivative of it may prove beneficial in reducing drug-resistant colon tumor hepatic metastases.

ACKNOWLEDGEMENTS

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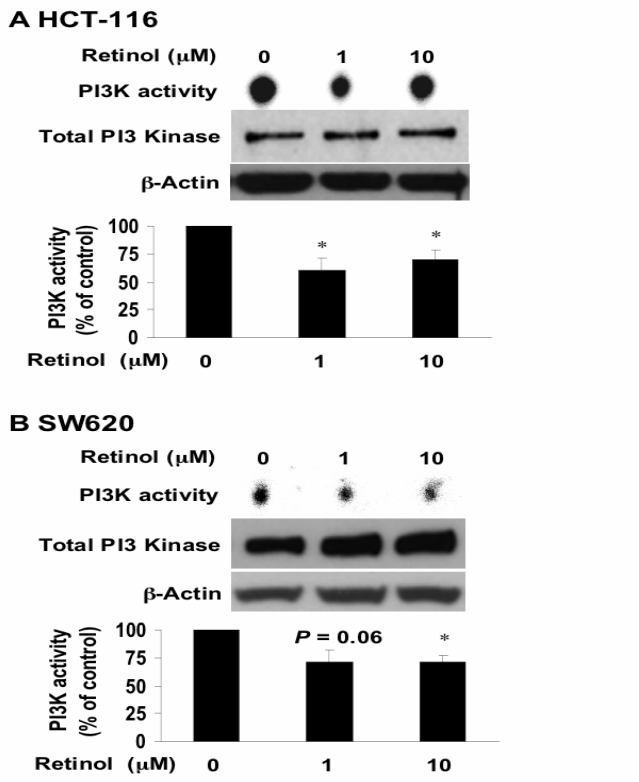

FIGURE 4.1. RETINOL DECREASES PI3K ACTIVITY. Total protein was isolated from HCT-116 (A) and SW620 (B) cells treated with 0 (vehicle control), 1, or 10 μM retinol for 30 min after 24 h of serum starvation. PI3K was immunoprecipitated using IRS-1 antibody as described in the Materials and Methods section. Immunoprecipitated PI3K was incubated with PI for 10 min followed by incubation with [γ-^{32}P] ATP for 20 min. Following incubation, the lipids were extracted and separated by TLC. PI3K activity was normalized to total PI3K protein. Total PI3K was detected using p85 antibody. Total PI3K protein was normalized to β-actin. Data are reported as mean ± SEM, of three independent experiments. *Significantly different from control ($P < 0.05$)

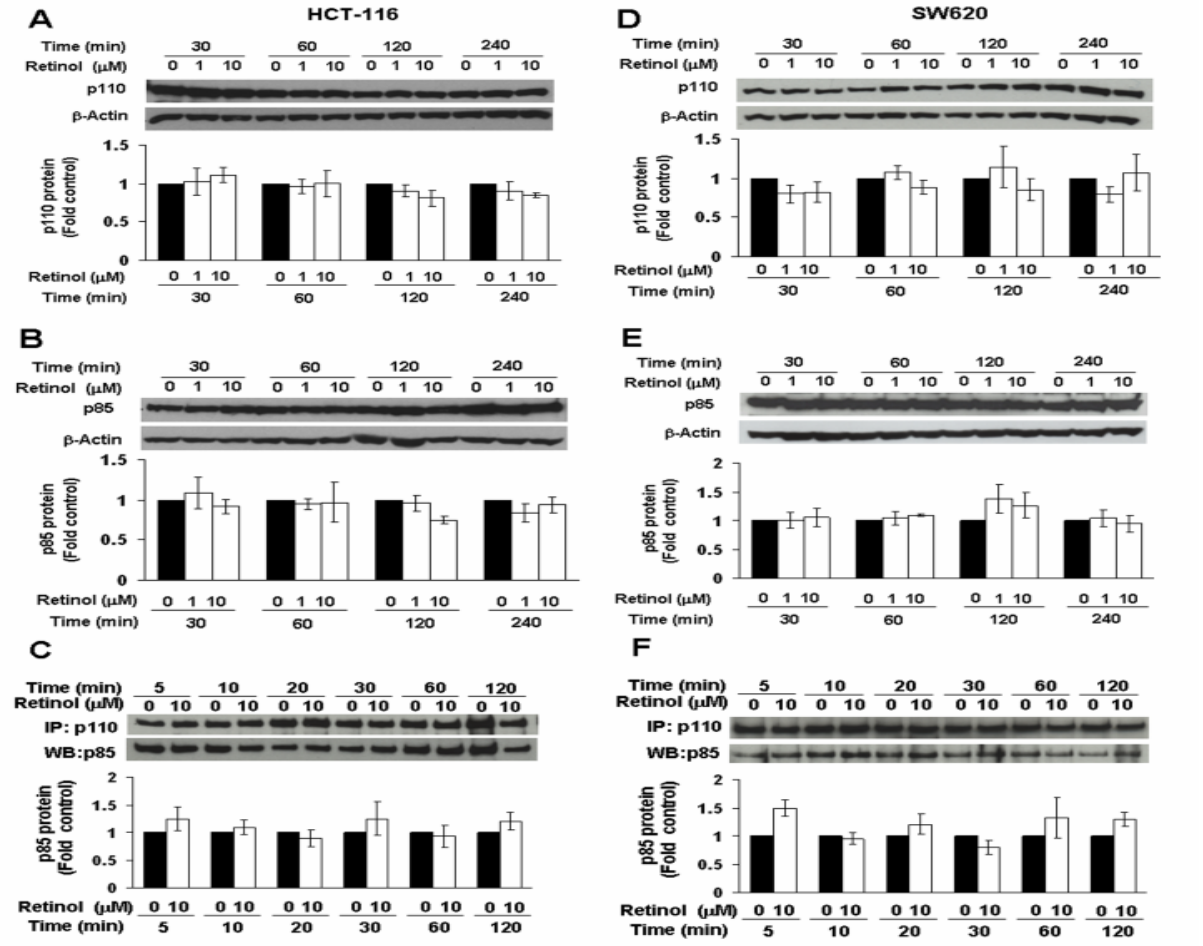

FIGURE 4.2. RETINOL DOES NOT CHANGE P110 OR P85 PROTEIN LEVELS OR P85/P110 HETERODIMERIZATION.

FIGURE 4.2. RETINOL DOES NOT CHANGE P110 OR P85 PROTEIN LEVELS OR P85/P110 HETERODIMERIZATION. Total protein was isolated from HCT-116 (A and B) and SW620 (D and E) cells treated with 0 (vehicle control), 1, or 10 μM retinol for 30 to 240 min after 24 h of serum starvation. p85 and p110 levels were detected using p85 and p110 antibodies as described in the Materials and Methods section. β-Actin was used as an internal loading control. Total protein was isolated from HCT-116 (C) and SW620 (F) cells treated with 0 (vehicle control) or 10 μM retinol for 5 to 120 min after 24 h of serum starvation. Total protein was immunoprecipitated using p110 antibody and sepharose 4A beads and detected using p85 antibody as described in the Materials and Methods section. Immunoprecipitated p110 was detected using p110 antibody and served as an internal loading control. Data are reported as mean ± SEM, of three independent experiments.

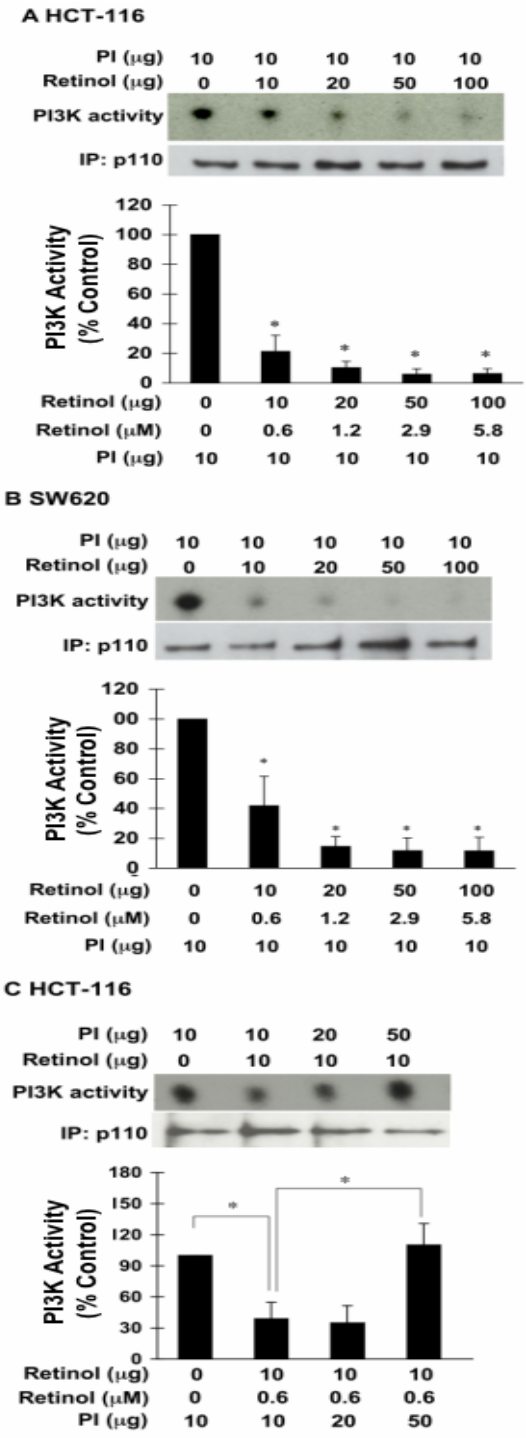

FIGURE 4.3. RETINOL DECREASES PI3K ACTIVITY BY INHIBITING PI3K AND PI INTERACTION.

FIGURE 4.3. RETINOL DECREASES PI3K ACTIVITY BY INHIBITING PI3K AND PI INTERACTION. Total protein was isolated from HCT-116 (A and C) and SW620 (B) cells treated with 10% FBS for 30 min after 24 h of serum starvation. Total PI3K was immunoprecipitated using p110 antibody as described in the Materials and Methods section. Immunoprecipitated PI3K was incubated with increasing concentrations of retinol and 10 μg PI for 10 min and then incubated with [γ-^{32}P] ATP for 20 min (A and B). Immunoprecipitated PI3K was incubated with increasing concentrations of PI and 10 μg retinol for 10 min and then incubated with [γ-^{32}P] ATP for 20 min (C). Total immunoprecipitated PI3K was detected by p110 antibody and used as an internal loading control. Following incubation the lipids were extracted and separated by TLC as described in the Materials and Methods Section. Data are reported as mean ± SEM, of three independent experiments. *Significantly different from control ($P < 0.05$).

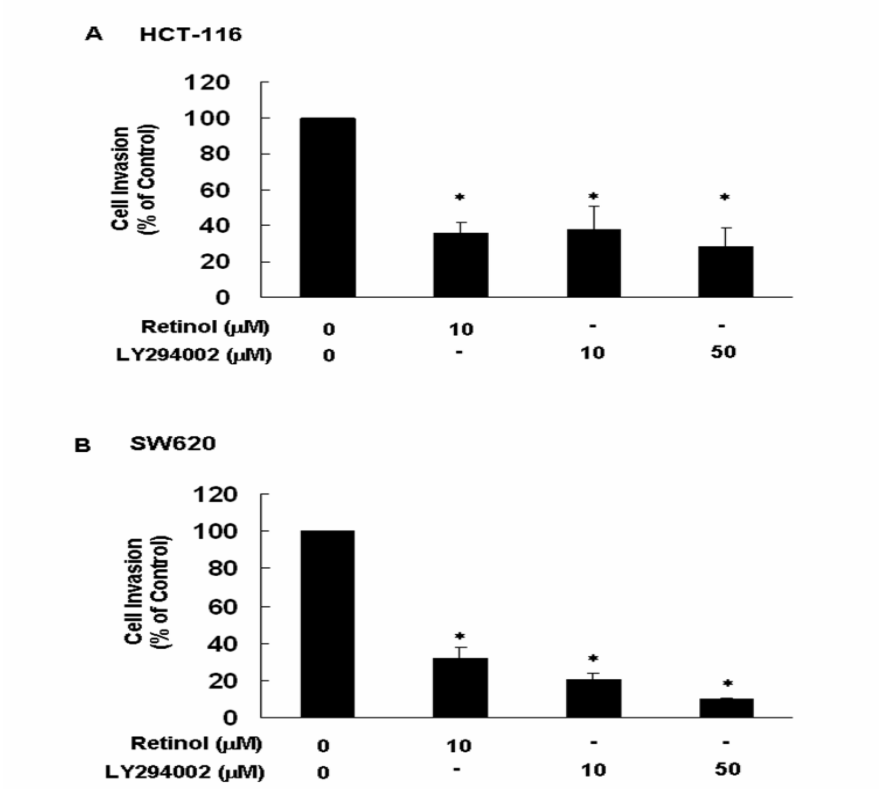

FIGURE 4.4. RETINOL AND LY294002 DECREASE CELL INVASION. HCT-116 (A) and SW620 (B) cells were serum starved for 24 h before seeding at a density of 1 x 10^5 cells/well on Matrigel-coated Boyden chambers. The upper portion of the chambers contained 0 or 10 μM retinol or 0, 10 or 50 μM LY294002 in serum free media. The lower chamber contained media supplemented with 10% FBS to serve as a chemoattractant. Cell invasion was measured after 24 h as described in the Materials and Methods section. Data are reported as mean ± SEM, of three independent experiments. *Significantly different from control ($P < 0.05$).

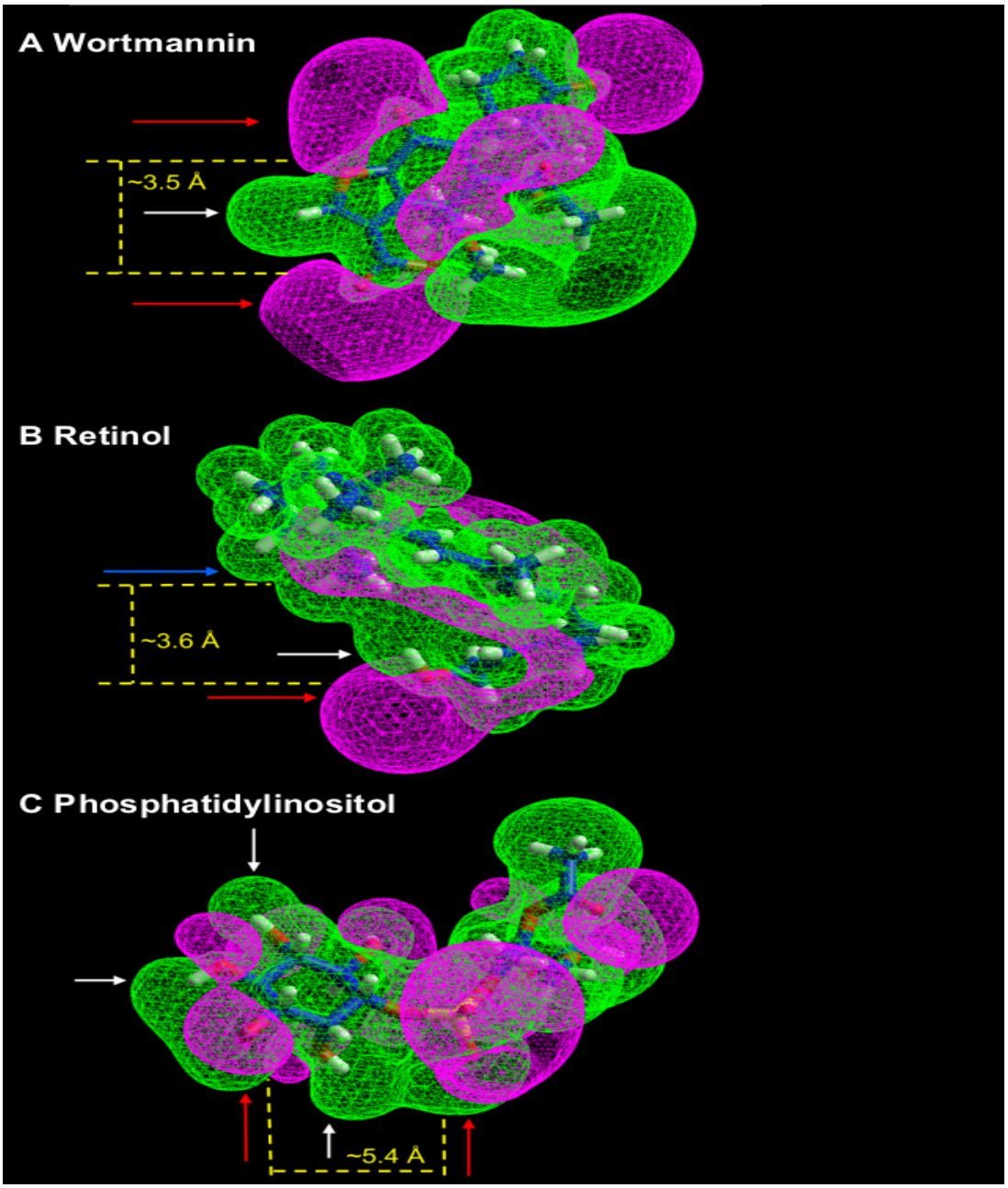

FIGURE 4.5. RETINOL AND WORTMANNIN EXHIBIT SIMILAR ELECTROSTATIC POTENTIAL SURFACES.

FIGURE 4.5. RETINOL AND WORTMANNIN EXHIBIT SIMILAR ELECTROSTATIC POTENTIAL SURFACES. Electrostatic potential surfaces for (A) wortmannin, (B) retinol and (C) a PI fragment were calculated as described in the Materials and Methods section. In all three panels purple wire frames indicate areas of negative electrostatic potential whereas green indicates positive potential. Surfaces are shown over a tube structure for the conformation with carbon atoms in blue, oxygen atoms in red, hydrogen atoms in white and phosphorus atoms in yellow. Arrows indicate areas associated with either reactive hydrogen atoms (white) or moieties responsible for largely negative electrostatic potential (red and blue).

Chapter 5: Dietary Vitamin A Decreases Liver Metastases of Colon Cancer in Mice

ABSTRACT

Previously, we showed that retinol inhibited *all-trans*-retinoic acid (ATRA)-resistant human colon cancer cell invasion via a retinoic acid receptor-independent mechanism *in vitro*. The objectives of the current study were to determine if dietary retinol inhibited metastasis of ATRA-resistant colon cancer cells in a nude mouse xenograft model. HCT-116, ATRA-resistant human colon cancer cells, were intrasplenically injected into female nude mice (BALB/cAnNCr-nu/nu). Injection of tumor cells resulted in the establishment of a splenic tumor which then shed cells that metastasized to the liver. Supplemental dietary vitamin A as retinyl palmitate was provided after injection for 5 wks in the chemotherapy study and for one month prior to and 5 wks after injection for the chemoprevention study. Consumption of a diet containing 200,000 IU vitamin A /kg diet decreased tumor incidence in the chemotherapy study and tumor multiplicity in the chemoprevention study. Supplementation with 200,000 IU vitamin A/kg diet decreased tumor incidence to 45.2% ($P = 0.14$) and tumor multiplicity to 17% ($P = 0.03$) of control in the chemotherapy and chemoprevention studies, respectively. Thus, supplemental dietary vitamin A may be an effective chemotherapeutic and chemopreventive agent to decrease the hepatic metastasis of colon cancer.

INTRODUCTION

Colorectal cancer is the third most common cancer and cause of death due to cancer in the United States. Death due to colorectal cancer is generally caused by distant metastasis rather than the primary tumor itself (7). Many cancers metastasize to specific organs [For a review please see: (172)]. For example, colon cancer mainly metastasizes to the liver and lungs (172). The diet contains (1) preformed vitamin A as retinyl esters in animal-derived food sources and (2) provitamin A carotenoids in plant-derived food sources. Once absorbed, retinol is re-esterified and transported to the liver, the major site of vitamin A storage, via chylomicrons. Hepatic retinol levels increase in response to supplementation and values in excess of 90 μM have been reported (44). Therefore, dietary vitamin A supplementation can increase retinol levels in the colon and liver, potentially affecting both primary colon tumors and those that have metastasized to the liver.

The retinoids, a group of compounds consisting of vitamin A (retinol), its natural metabolites and several synthetic compounds, have also been shown to inhibit metastasis in a variety of model systems. For example, dietary retinyl palmitate decreased malignant melanoma metastasis in mice (86). ATRA decreased breast cancer (105), gastric cancer (87) and colon cancer cell invasion (88) *in vivo* and rhabdomyosarcoma metastasis in rats (135). Also, retinol decreased hepatic metastases in a hamster model of pancreatic ductal carcinoma (173). Previously, we showed that retinol decreased the growth and invasion of ATRA-resistant human colon cancer cells via a novel RAR-independent mechanism *in vitro* (144,162,174).

Recent studies in our laboratory showed that retinol decreases MMP-2, -9 and PI3K activity to inhibit cancer cell invasion *in vitro* (144,174) In addition, nude mice studies bearing colon cancer cells to generate liver metastasis showed that down regulation of these proteins was associated with regression of cancer progression. For example, interferon-α administration to nude mice following intrasplenic injection with human colon cancer cells (KM12L4) showed a decrease in MMP-9 and basic fibroblast growth factor (bFGF) levels in liver metastatic tumors and reduced liver metastasis incidence (175). PI3K specific siRNA treatment suppressed the hepatic metastases of colon cancer cells injected intrasplenically (176). On the other hand, an HCT-116 mutant cell line expressing constitutively active PI3K promoted liver metastases in orthotopically implanted nude mice than when compared to nude mice implanted with wild type HCT-116 cells (24).

The objectives of the present study were to determine if dietary vitamin A supplementation decreased the hepatic metastases of colon tumor cells *in vivo*. Liver metastases were induced by intrasplenic injection of wild type HCT-116 human colon carcinoma cells into female BALB/cAnNCr-nu/nu nude mice. Hepatic tumor incidence and multiplicity were measured to examine the effect of dietary vitamin A supplementation. MMP-2, -9 and phospho-Akt levels were also examined to determine the mechanism by which dietary vitamin A reduces liver metastasis *in vivo*.

MATERIALS AND METHODS

Tissue Culture

The human colorectal carcinoma cell line, HCT-116 was obtained from the American Type Culture Collection (Manassas, VA) and grown in DMEM in a humidified atmosphere at 37°C with 5% CO_2. Media were supplemented with 10% FBS (fetal bovine serum) and antibiotics (1000 U/mL penicillin and 1000 μg/mL streptomycin).

Animal Studies

This animal study was performed in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory animal under assurance number A4107-01 and with the University of Texas IACUC, protocol number 05060201.

Metastasized liver tumors were generated by intrasplenic injection of 2 x 10^6 HCT-116 cells in 50 μl of magnesium and calcium free HBSS into female BALB/cAnNCr-nu/nu mice (Strain code 01B70, NCI-Frederick, Frederick, Maryland). Purified AIN-76 rodent diet was modified to contain different concentration of retinyl palmitate and irradiated (Research Diets, Inc). This modified AIN-76 diet contains 2,400 (control-meets National Research Council (NRC) mouse vitamin A requirement (177)), 12,000, 25,000, 50,000, 100,000 or 200,000 IU vitamin A/kg diet as retinyl palmitate. Diet compositions are described in Table 5.1. Diets were stored at 4 °C in black painted containers. Diets and water were consumed *ad libitum.* Fresh diets were

provided every other day and feed consumption was recorded at this time. The mice were weighed weekly.

Chemotherapy experiment:

After tumor cell injection, mice (n=15 per group) aged 6 to 8 weeks were randomly assigned to diets containing 2,400, 12,000, 25,000, 50,000, 100,000 or 200,000 IU vitamin A/kg diet as retinyl palmitate. These diets provided 1, 5, 10, 20, 40 and 80 X the daily vitamin A requirement of mice. Diets were supplied immediately after surgery and consumed for five weeks, until sacrifice.

Chemoprevention experiment:

Mice (n=14 per group) between aged 6 to 8 weeks were fed either the 2,400 IU vitamin A/kg control diet or a diet containing 200,000 IU vitamin A/kg diet as retinyl palmitate for four weeks before surgery. After surgery, the mice consumed the same diets for five more weeks prior to sacrifice to examine liver metastases.

Immunohistochemistry

Livers were extracted, fixed with 10% buffered formaldehyde and paraffin-embedded. Cytokeratin (CK) 20 (sc-17113, Santa Cruz Biotechnology, Santa Cruz, CA), a protein expressed in human goblet cells and enterocytes (178), was used to detect metastasized human colon cancer cells in paraffin-embedded liver sections. For the chemotherapy experiment, two random slides were stained with CK 20 from each liver to determine the presence of microscopic liver metastasis of colon cancer cells. If both

slides showed negative staining, four more random slides were selected and stained with CK20 to confirm negative staining.

MMP-2 (AB19167, Chemicon, Temecula, CA), MMP-9 (AB16306-50, Abcam, Cambridge, MA), total Akt (#4691, Cell Signaling, Beverly, MA), and phospho-Akt (Ser 473) (sc-7985-R, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were also used for immunohistochemistry. Three consecutive 4 μm sections were stained with CK20, MMP-2, and MMP-9 or CK20, total Akt, and phospho-Akt in this order. Tissue sections were deparaffinized in xylene, and rehydrated in descending ethanol series to water. Next, endogenous peroxidase was blocked by incubating with 3% hydrogen peroxide for 10 min. Antigen retrieval was performed by heating tissue sections in 10 mM citrate buffer (pH 6.0) for 10 min in a microwave followed by a 20 min cool down. Non-specific antibody binding was blocked with Biocare Blocking Reagent (BS966M, Biocare, Phoenix, AZ) for 10 min. A 1:100 dilution of CK20, a 1:100 dilution of MMP-2, a 1:500 dilution of MMP-9, a 1:100 dilution of total Akt and a 1:50 dilution of phospho-Akt were used for primary antibody incubation. The samples were then incubated with horseradish peroxidase conjugated anti-rabbit (#RMR622, Biocare, Concord, CA) or anti-goat (#GHP516L, Biocare) secondary antibodies. The slides were the counterstained, mounted and observed by light microscopy. All immunohistochemistry was performed by the Histology & Tissue Processing Facility Core in the University of Texas M.D. Anderson Cancer Center.

Statistical Analysis

Values shown are the mean ± SEM. Statistical tests other than tumor incidence were performed using SPSS (Apache Software Foundation, Wilmington, DE, version 12.0 for Windows). Fisher's exact test was used for the statistical analysis for tumor incidence. Body weight, food intake, and tumor multiplicity were analyzed using two-tailed *t*-tests comparing each vitamin A concentration to control (2,400 IU/kg). Results were considered significantly different at $P < 0.05$.

RESULTS

Food intake and Body Weight Analysis

At the beginning of the chemotherapy study, each group of mice (n=15 per group) had a mean body weight of 17.7 ± 0.02 g. Five weeks after tumor injection, the average body weight of the group consuming the diet containing 200,000 IU vitamin A/kg (17.7 ± 0.01 g) diet was less than that of the mice consuming the control diet (19.4 ± 0.12 g) (Fig. 5.1A). After consuming the diets for nine weeks, there were no significant differences in body weight between control mice, and those consuming 200,000 IU vitamin A/kg diet in the chemoprevention study. No mice in the chemoprevention study lost weight; both groups gained 4.8 ± 0.13 g of body weight. However, most weight gain was before tumor injection.

There were no differences in food intake among the different dietary groups for both the chemotherapy and chemoprevention experiments. However, the amount of

food intake decreased after tumor injection in both experiments (Fig. 5.1 B and D). In addition, only one mouse in the chemotherapy study, consuming 200,000 IU vitamin A/kg diet, showed skin redness and desquamation. No mice in the chemoprevention study showed any signs of vitamin A toxicity. These data show that high levels of vitamin A supplementation do not adversely affect body weight and food intake or cause toxicity in mice.

The effect of retinol on liver metastases incidence in the chemotherapy study.

Because visible metastases were not always apparent and to confirm that the visual metastases were indeed true metastases of human origin, liver sections were subjected to immunohistochemical analysis for the presence of CK 20, a marker of human colon cells. This also allowed us to visualize micrometastases. As can be seen in Fig. 5.2A, dietary supplementation with 50,000, 100,000 or 200,000 IU vitamin A/kg diet decreased the incidence of metastasis. The number of metastases per dietary group is displayed in Table 5.2. The greatest degree of inhibition was achieved by the consumption of the 200,000 IU vitamin A/kg diet. These mice exhibited a metastasis rate 45.2% less ($P = 0.14$) than that exhibited by mice consuming the control diet (Table 5.2 and Fig. 5.2C). These data indicate that dietary supplementation with vitamin A trends to inhibit the metastases of human colorectal cancer cells in a nude mouse xenograft chemotherapy model, confirming our *in vitro* observations.

The effect of retinol on tumor multiplicity in chemoprevention study.

Because the incidence of metastasis was very high and visual tumors were apparent in the chemoprevention study, we determined metastasis multiplicity, defined here as the number of metastatic tumors per liver, per mouse. Supplementation with 200,000 IU vitamin A/kg diet significantly reduced tumor multiplicity from 56.57 ± 21.99 to 9.86 ± 3.8 (P = 0.03) (Fig. 5.3) which decreased tumor multiplicity to 17% of control. These data indicate that dietary vitamin A supplementation, prior to tumor cell injection, reduces the number of metastases per liver.

In a previous study, we showed that retinol decreased MMP-2, -9 and PI3K activity resulting in inhibition of human colon cancer cell invasion *in vitro* (144,174). To determine if the activity of these enzymes were decreased by dietary vitamin A supplementation, liver metastases were stained with MMP-2, -9, total Akt, and phospho-Akt. Phospho-Akt corrected for total Akt represents PI3K activity. These four antibodies were pre-examined for positive staining in different tissues (data not shown). Liver metastases introduced by intrasplenic injection of human colon cancer cells showed no staining for MMP-2 and -9 (Fig. 5.4 B and C). Metastasized tumors showed positive total and phospho-Akt staining. However, vitamin A supplementation did not change the level of total or phospho-Akt (Fig. 5.4 D to F).

DISCUSSION

The liver is the major storage site for vitamin A and the target organ for colon cancer metastasis. Previously, we showed that retinol decreased ATRA resistant colon

cancer cell growth and invasion *in vitro* (144,162,174). Here, we demonstrate that 200,000 IU vitamin A/kg diet decreased the incidence and multiplicity of liver metastases in nude mice intrasplenically injected with ATRA-resistant human colon cancer cells (Table 5.2, Fig.5.2 and Fig. 5.3). Previously, we showed that retinol decreased ATRA-resistant colon cancer invasion by inhibiting MMP-2, -9 and PI3K activity *in vitro* (144,174). However, MMP-2 and -9 were not expressed by liver metastases and PI3K activity in liver metastases was not changed by dietary vitamin A supplementation (Figure 5.3).

The recommended daily allowance (RDA) levels for vitamin A is 2333 IU/day for female adult humans (over age 14) (179). Daily ingestion of over 100,000 IU (33.3X the RDA) for more than six months is considered toxic [For a review please see; (180)]. In addition, the smallest daily vitamin A uptake reported to cause liver cirrhosis was 25,000 IU (10X the RDA) for six years (181). The recommended vitamin A dietary level from the NRC for mice is 2,400 IU of vitamin A/kg of diet (182). The diets used in this study contain 2,400 (1X NRC), 12,000 (5X NRC), 25,000 (10X NRC), 50,000 (20X NRC), 100,000 (40X NRC) and 200,000 (80X NRC) IU vitamin A/kg diet. However, only 200,000 IU/kg tended to decrease tumor incidence and significantly decreased tumor multiplicity in the chemotherapy and chemoprevention studies, respectively. If the NRC recommendation for mice is proportional to the RDA for humans, only 12,000 IU/kg is in the safe range for vitamin A toxicity. The amount of vitamin A which showed adverse effect (200,000 IU/kg, 80X RDA) on liver metastasis might be harmful for humans. There is a study showing that ingestion of 200,000 IU (87X NRC) vitamin A/kg diet decreased aberrant crypt foci incidence in rat (2300 IU/kg

– NRC rat vitamin A requirement) (45). However, this study did not mention anything concerning toxicity caused by 200,000 IU/kg of vitamin A supplementation. We did not use different diets between 12,000 and 100,000 IU/kg in the chemoprevention study. Therefore, it is possible that a lower level of vitamin A supplementation would show the same effect as 200,000 IU/kg vitamin A supplementation and decreases metastasis multiplicity in chemoprevention study.

Although dietary vitamin A decreased tumor incidence in chemotherapy study, the tumor incidence in the chemoprevention study was 100% regardless of vitamin A supplementation level. It is important to note that different cell preparations were used for the two dietary experiments, therefore direct comparisons between experiments may not be possible. In each case, the viability of the HCT-116 cell suspension was assessed before and after surgery via trypan blue dye exclusion assay. Cell viability following surgery was > 92% in both cases. We believe the higher incidence of metastasis in the chemoprevention experiment most likely reflects the cell preparation injected. Alternatively, Vitamin A might show a different effects depending on time administered relative to tumor injection. For example, folate, a promising micronutrient for colon cancer prevention, inhibits the initiation of colon cancer progression (28). However, folate deficiency also reduces the size of colon cancer tumors (28), indicating that the same nutrient can show two different effects depending on the stage of colon cancer. Therefore, it is possible that preloading vitamin A in the liver might promote tumor progression but decrease tumor growth.

We previously showed that retinol decreased the invasion of ATRA-resistant human colon cancer cells by decreasing MMP-2, -9 and PI3K activity *in vitro*.

However, we did not observe MMP-2 and -9 staining in the metastatic liver tumors in the current study. These tumors were derived from the same cell line, HCT-116, used our *in vitro* study. We hypothesized that the effect of vitamin A on the activity of these proteins may be dependent upon stage of metastasis. For example, MMP-2 and -9 may required at the beginning of liver metastases. Therefore, mice may need to be sacrificed at an earlier time point to determine changes in MMP-2 and -9 protein levels in response to dietary vitamin A supplementation.

We detected phospho-Akt staining in liver metastases of human colon cancer cells, however, the level of phospho-Akt was not changed by vitamin A supplementation. Rychahou *et al* showed that inhibition of PI3K subunits, either p85 or p110, using siRNA decreased tumor multiplicity (176). In the Rychahou *et al* study, they also examined the expression of Akt1 and Akt 2. In human, there are three isoforms of Akt, designated Akt1, 2, and 3. Previous studies showed increased Akt 1 and 2 activation in colorectal cancers and colon cancer cell lines (183,184). The Rychahou *et al* study showed Akt1 expression was highly variable but increased expression of Akt 2 was found in all human colorectal cancers especially in the late stage of cancers. Samuels *et al* showed that Akt1 is the predominant form of Akt in HCT-116 cells (185). However, Bruzek *et al* showed expression of both Akt 1 and 2 in the same cell line (186). Akt3 expression has not been studied in this cell line. The contribution of Akt1 and Akt2 to cell invasion is currently unclear in the HCT-116 cell line. Both total and phospho-Akt antibody used in this study detect all three types of Akt. Therefore, we might not detect the changes of activation of the specific isoform of Akt which is important for liver metastasis of colon

cancer. Therefore, determination of the Akt isoform required for invasion should occur before additional investigation of PI3K activity in liver metastasis.

In conclusion, the present study shows that dietary vitamin A supplementation decreases tumor incidence in mice subjected to a chemotherapy dietary regimen and tumor multiplicity in mice subjected to chemoprevention regimen. However, the inhibition of MMP-2, -9 and PI3K activity by retinol treatment showed in our previous *in vitro* studies was not observed here. To our knowledge, this study is the first to show that dietary vitamin A supplementation inhibits colon cancer metastasis to the liver in a mouse model. Taken together, these data suggest the possibility of dietary vitamin A supplementation for colon cancer therapy and prevention.

ACKNOWLEDGEMENT

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Table 5.1. Diet composition

Dietary vitamin A (IU/kg)	2400		12,000		25,000		50,000		100,000		200,000	
	gm%	kcal%	gm%	kcal%	gm%	kcal%	gm%	kcal%	gm%	kcal%	gm%	kcal%
Protein	20	21	20	21	20	21	20	21	20	21	20	21
Carbohydrate	66	68	66	68	66	68	66	68	66	68	66	68
Fat	5	12	5	12	5	12	5	12	5	12	5	12
kcal/gm ,Total	3.90	100.0	3.90	100.0	3.90	100.0	3.90	100.0	3.90	100.0	3.90	100.0
Ingredient	gm	kcal	gm	kcal	gm	kcal	gm	kcal	gm	kcal	gm	kcal
Casein	200	800	200	800	200	800	200	800	200	800	200	800
DL-Methionine	3	12	3	12	3	12	3	12	3	12	3	12
Corn Starch	150	600	150	600	150	600	150	600	150	600	150	600
Sucrose	500	2000	500	2000	500	2000	500	2000	500	2000	500	2000
Cellulose,BW200	50	0	50	0	50	0	50	0	50	0	50	0
Corn oil	50	450	50	450	50	450	50	450	50	450	50	450
Mineral Mix S10001	35	0	35	0	35	0	35	0	35	0	35	0
Vitamin Mix V13001, no added Vitamin A	10	40	10	40	10	40	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0	2	0	2	0	2	0
Retinyl Palmitate, 250,000 IU/gm	0.0096	0	0.048	0	0.1	0	0.2	0	0.4	0	0.8	0
Total	1000.0596	3902	1000.098	3902	1000.15	3902	1000.25	3902	1000.45	3902	1000.85	3902

Table 5.2. Dietary vitamin A content versus incidence of metastases

Dietary Vitamin A (IU/kg diet)	Number of mice /group with metastases	Tumor incidence (% of control)
2,400 (control)	10/15	100
12,000	11/15 ($P = 1.00$)	110
25,000	11/15 ($P = 1.00$)	110
50,000	8/15 ($P = 0.71$)	72.6
100,000	8/15 ($P = 0.71$)	72.6
200,000	5/15 ($P = 0.14$)	45.2

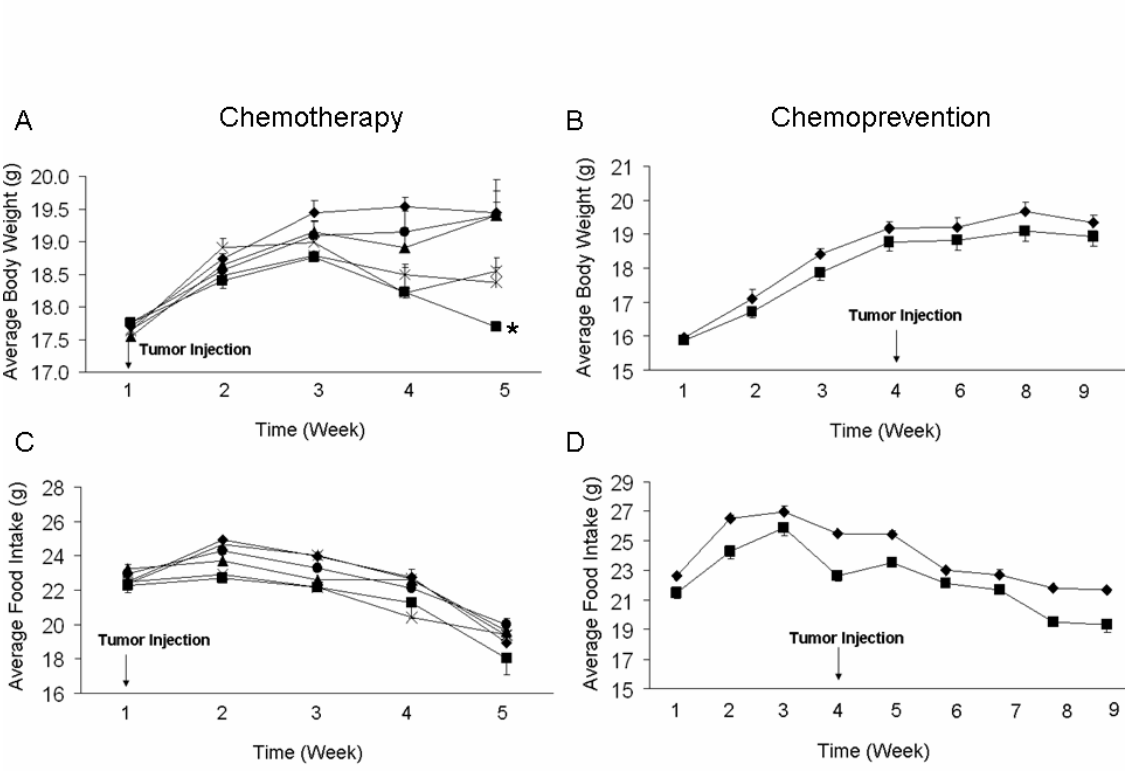
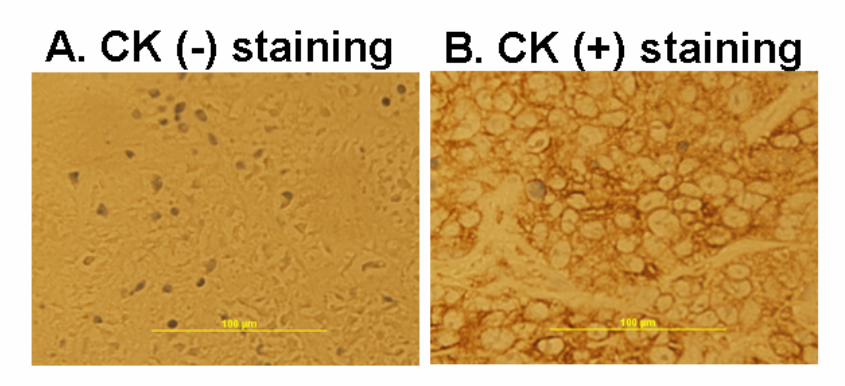


FIGURE 5.1. AVERAGE FOOD INTAKE AND BODY WEIGHT. Average body weight for 5 weeks on vitamin A supplemented diets of the chemotherapy study (A) and for 9 weeks on diets of the chemoprevention study (B). Average food intake for the chemotherapy study (C) and the chemoprevention study (D). Mice were fed with 2,400 (♦), 12,000 (▲), 25,000 (●), 50,000 (X), 100,000 (*), and 200,000 (■) IU vitamin A/kg diet in the chemotherapy and 2,400 (♦), and 200,000 (■) IU vitamin A/kg diet in the chemoprevention sutdy. Data shown are mean ± SEM [n=15 (A and B) and n=14 (C and D)]. *Significantly different from control ($P < 0.05$).



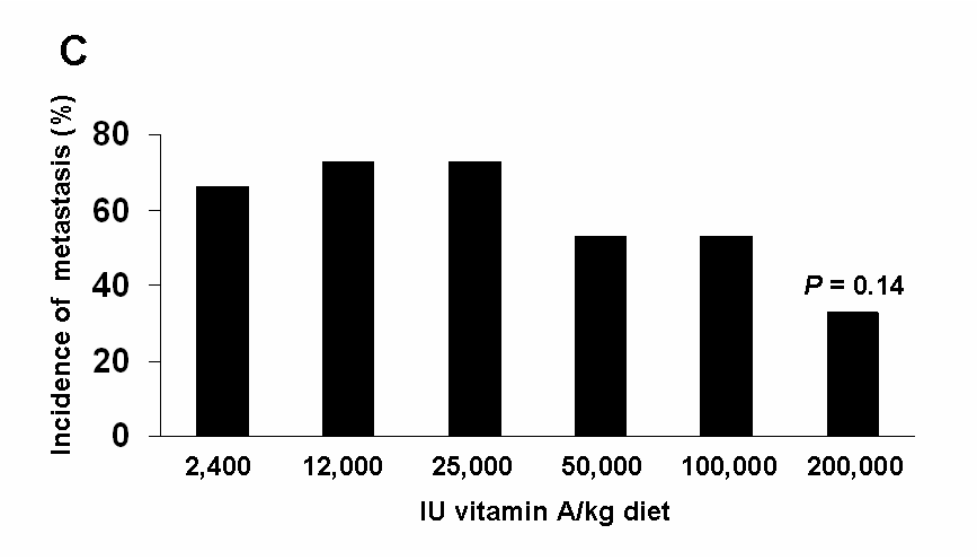


FIGURE 5.2. DIETARY VITAMIN A SUPPLEMENTATION DECREASES METASTATIC INCIDENCE; CHEMOTHERAPY DIETARY MODEL. Ninety female BALB/cAnNCr-nu/nu mice, aged 6 to 8 wks, were intersplenically injected with 2 x 10^6 HCT-116 cells in 50 µl of magnesium and calcium free HBSS. Immediately following surgery the mice were divided into six groups (n=15 per group) and consumed diets containing increasing amounts of vitamin A as retinyl palmitate. Five weeks following surgery, all mice were sacrificed and the incidence of liver metastases assessed by cytokeratin 20 staining, (A) Negative (from normal liver) and (B) positive (liver metastases) CK staining. Dietary supplementation with 50,000, 100,000 or 200,000 IU vitamin A/kg diet decreased the incidence of hepatic metastasis (C). Data shown are % of mice per group exhibiting metastasis.

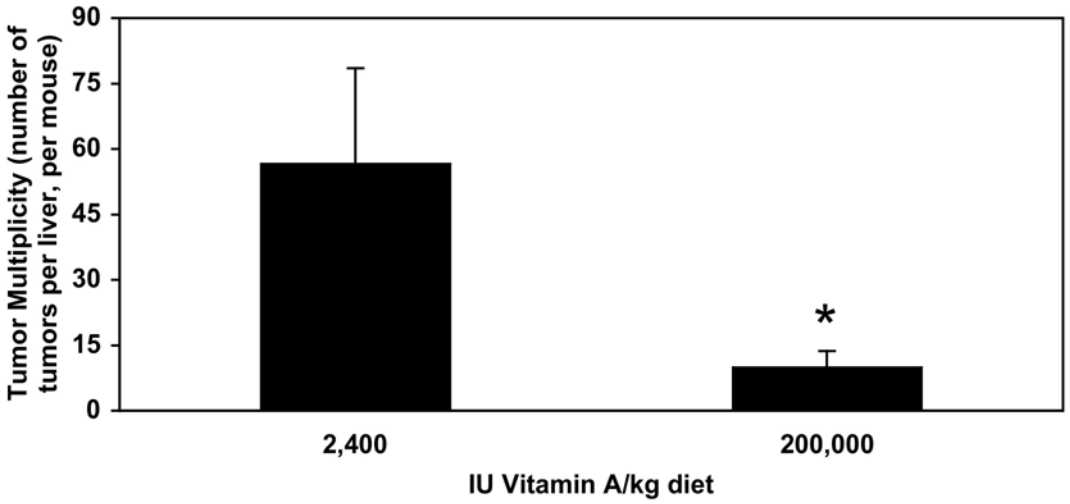


FIGURE 5.3. DIETARY VITAMIN A SUPPLEMENTATION REDUCES TUMOR MULTIPLICITY IN MICE SUBJECTED TO THE CHEMOPREVENTION DIETARY REGIMEN. BALB/cAnNCr-nu/nu female mice, aged 6 to 8 wks, were randomly divided into two groups. The first group consumed the control diet, containing 2,400 IU vitamin A/kg diet. The second group consumed a diet supplemented with 200,000 IU vitamin A/kg diet. The mice consumed these diets *ad libitum* for one month prior to surgery. To generate metastases, the mice were intersplenically injected with 2 x 10^6 HCT-116 cells in 50 μl of magnesium and calcium free HBSS. Following surgery, the mice continued to consume their respective diets for five weeks. After five weeks, all mice were sacrificed and their livers examined visually for the presence and number of metastases. Data shown are mean ± SEM, n=14. *Significantly different from control ($P < 0.05$).

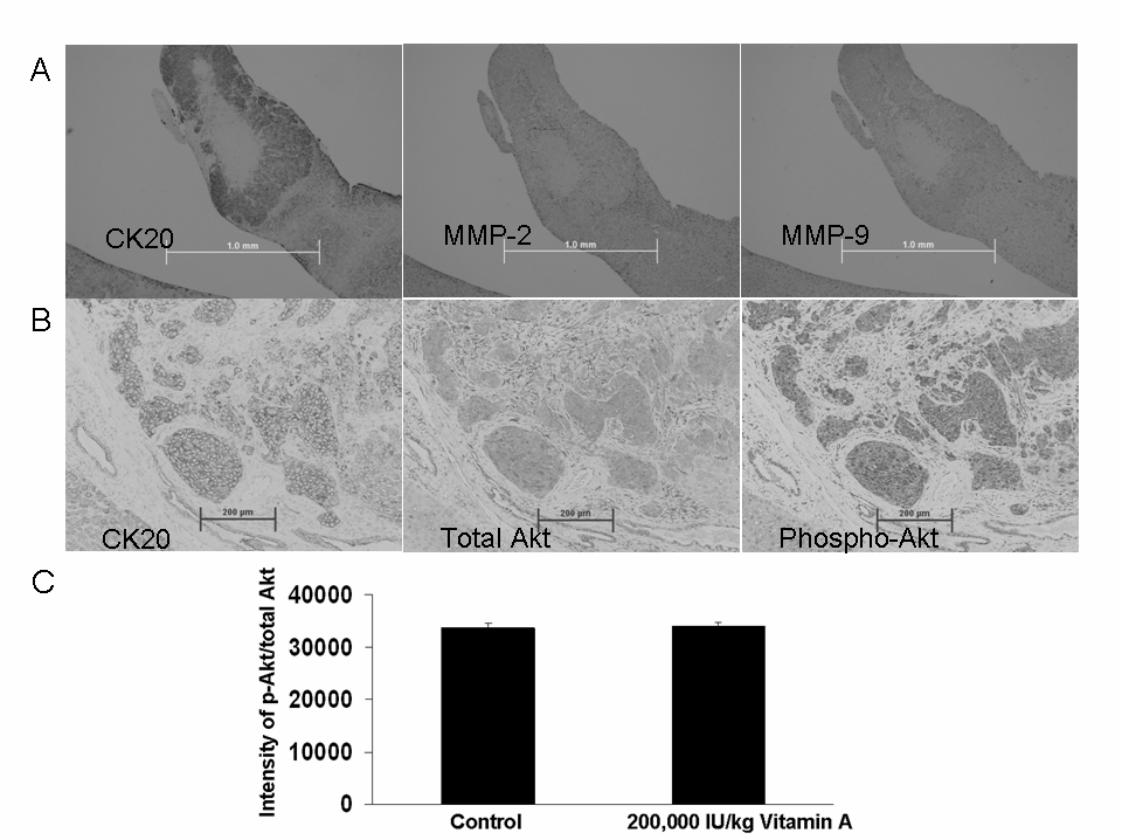


FIGURE 5.4. IMMUNOHISTOCHEMICAL DATA FROM CHEMOPREVENTION STUDY. Consecutive slides of liver metastatic tumor were stained with CK20, MMP-2, and MMP-9 (A). In addition, consecutive slides of liver metastasis sample were stained with CK20, total Akt, and phospho-Akt (B). One representative sample is shown. The level of active Akt was not altered by vitamin A supplementation (C). Data shown are mean ± SEM, n=5.

Chapter 6: Summary and Future Directions

Colorectal cancer is the third most common cancer in the USA. Early diagnosis and treatment helps decrease the five year survival rate for patients with colorectal cancer. However, colorectal cancer is still the third leading cause of cancer death in the USA. Moreover, distant metastases of colon cancer are more problematic. These facts indicate that we need more effective treatment for colon cancer. In addition, many studies have been conducted to show that micronutrients reduce colon cancer incidence and progression. We focused on the effect of retinol on colon cancer progression because colonocytes are primarily exposed to retinol from the diet. Chapter 1 introduced the importance of inhibiting human colon cancer cell growth and metastasis. It also provided the reasons to study the effect of retinol on colon cancer growth and metastasis instead of other bioactive retinoids such as ATRA.

Chapter 2 showed that retinol inhibited the growth of both ATRA-sensitive and ATRA-resistant human colon cancer cell lines independent of the ATRA/RAR/RARE retinoid signaling pathway. First, retinol was not metabolized to other bioactive retinoids, such as ATRA. Second, retinol did not activate RARE-mediated gene transcription. Finally, a RAR-antagonist blocked the ability of ATRA to inhibit the growth of ATRA-sensitive HCT-15 cells, as expected, but did not block the ability of retinol to inhibit the growth of any cell line examined. Therefore, even in the presence of functioning RAR, retinol did not inhibit cell growth by the actions of its metabolite ATRA, because this metabolite is not present in ATRA-sensitive HCT-15 cells. In addition, retinol acted through a novel mechanism to inhibit the growth of both ATRA-

sensitive and ATRA-resistant colon cancer cells by affecting cell cycle progression. In Chapter 2, retinol also decreased tumor cell growth by inducing $G_{0/1}$ arrest in three of the cell lines examined.

These results suggest future directions concerning the molecular mechanisms underlying retinol's ability to arrest the cell cycle independent of RAR/RXR/RARE and inhibit cancer cell growth. Progression from $G_{0/1}$ to S phase is mediated by cyclins D and E and cyclin dependent kinases (Cdk) 2, 4 and 6. Cyclin-Cdk complexes are influenced by several cell cycle regulatory proteins such as protein kinases and phosphatases that modify Cdks, the cyclin dependent kinase inhibitors (CKIs); p27, p21 and p16, and regulatory proteins such as p53 and E2F. Unfortunately, the effect of retinoids on cell cycle regulatory proteins appears to be cell type specific (30). For example, cyclin D1 was responsible for G_1 arrest in carcinogen-exposed immortalized human bronchial epithelial cells (68,69). In contrast, decreased phosphorylation of pRB was the major factor for $G_{0/1}$ arrest in MCF-7 breast cancer cells (70-72). We have seen that inhibition of invasion by retinol treatment depends on posttranslational modification such as phosphorylation events (Chapters 3 and 4). However, we have not examined the effect of cycloheximide (translation inhibitor) and actinomycin D (transcription inhibitor) on retinol's inhibitory effect on cell growth due to toxicity of these chemicals when treating for more than 24 hr required for retinol to show its inhibitory effect on cancer cell growth. Therefore, the inhibitory effect of retinol on colon cancer cell growth is open to all three possibilities; (1) inhibiting mRNA or (2) protein synthesis or (3) inhibiting posttranslational modification. To examine possible candidates for cell cycle

arrest-related proteins affected by retinol, we could used a cell cycle microarray as a start to narrow down target molecules.

Chapters 3, 4 and 5 demonstrated that retinol decreased the invasion of human colon cancer cell lines *in vitro* and *in vivo*. Figure 6.1 summarizes retinol's inhibitory effects on colon cancer metastasis *in vitro* (Chapters 3 and 4). Chapter 3 showed that retinol inhibited ATRA-resistant colon cell invasion by decreasing gelatinase activity and increasing TIMP-1 levels *in vitro* through an ATRA and RAR-independent mechanism. Chapter 4 demonstrated that retinol treatment decreased PI3K activity in ATRA-resistant human colon cancer cell lines. Retinol decreases PI3K activity by acting similarly to the known PI3K inhibitor, wortmannin, not due to reduced p85 regulatory subunit or p110 catalytic subunit levels or to a decrease in the heterodimerization of these two proteins. Chapter 5 showed that dietary vitamin A supplementation had both chemopreventive and chemotherapeutic potential to decrease liver metastases of colon tumor cells in a nude mice xenograft model.

We have studied the upstream signaling molecule (PI3K) and downstream effecter molecules (gelatinases and TIMP-1) affected to retinol to inhibit colon cancer cell invasion. However, it is necessary to link PI3K to the gelatinases and TIMP-1. The PI3K/Akt signaling pathway up-regulates metastasis related genes including MMP-2 and –9 in breast cancer and colon cancer [for review see: (9,164)]. Unfortunately, the molecular mechanisms are not well understood between activation of PI3K and activation of MMP-2 and -9 activity (187,188). Our studies showed that retinol decreased MMP-2 and –9 activity (chapter 3) and PI3K activity (chapter 4). Therefore, future studies

could investigate the molecular mechanisms or signaling pathways between PI3K and MMP-2 and -9 regulated by retinol that ultimately decrease cell invasion.

In addition, we expected to see changes in protein levels of MMP-2 and -9 from the IHC experiment in Chapter 5. However, MMP-2 and -9 showed negative staining in liver metastases. As mentioned in the discussion in Chapter 5, MMP-2 and -9 may be required in the early stages of tumor metastasis. Therefore, in a future experiment, tumor-injected mice should be sacrificed at earlier times to detect changes in MMP-2 and -9 expression. Finally, different concentrations of vitamin A in diets between 12,000 to 100,000 IU/kg should be used in the chemoprevention study to determine the minimum amount of vitamin A supplementation which will decreases liver metastasis.

In conclusion, the first part of my work (Chapter 2) shows that retinol acts through a novel mechanism to inhibit the growth of both ATRA-sensitive and ATRA-resistant colon cancer cells by affecting cell cycle progression. In addition, the second part of my work (Chapters 3 to 5) concerns the ability of retinol to decrease the metastasis of ATRA-resistant colon cancer *in vivo* and *in vitro*. To our knowledge, these studies are the first to show that retinol inhibits MMP-2 and -9 activity and PI3K activity to decrease cancer cell invasion *in vitro*. There are many preclinical studies to support the inhibitory effect of vitamin A supplementation on many other types of cancer progression; however, this is the first study to show that vitamin A supplementation decreases the liver metastases of colon cancer. My work suggests that dietary vitamin A supplementation will prevent colon cancer progression.

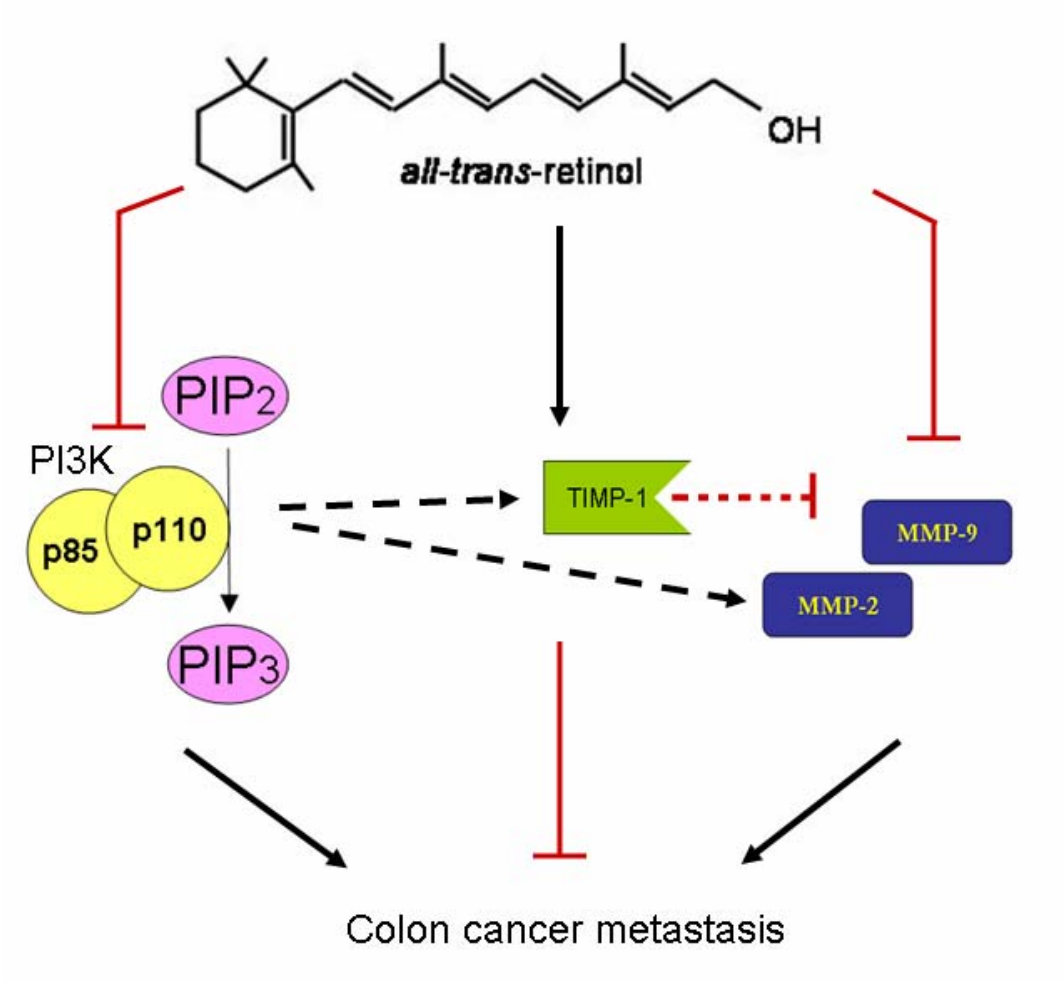


FIGURE 6.1. RETINOL DECREASES PI3K ACTIVITY AND/OR MMP-2 AND -9 ACTIVITY AND INCREASES TIMP-1 SECRETION TO INHIBIT COLON CANCER METASTASIS. Red blunt arrows indicate the inhibition and black arrows indicate activation. Red and black dashed arrows indicate unsolved relationships between each element.

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